# Daily Heat Stress Induces Accumulation of Non-functional PSII-LHCII and Donor-side Limitation of PSI via Downregulation of the Cyt $b_6f$ Complex in *Arabidopsis thaliana*

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## Abstract

Ongoing climate change has intensified heatwaves, which suppress photosynthesis and thus threatens agriculture. While acute heat stress damages photosystem II (PSII), detach light harvesting complex (LHC)II from PSII core and linearize thylakoid membrane, the consequences of prolonged recurring moderate heat exposure across the plant life cycle remain unclear. Here we characterized the effects of long-term daily exposure to high temperature on thylakoid membrane ultrastructure, photosynthetic protein complexes and their function, as well as to the redox states of PSI electron donors and acceptors in *Arabidopsis thaliana*. We show that plants exposed daily to 38°C for four hours exhibit altered thylakoid ultrastructure and structurally intact, but functionally impaired PSII-LHCII. Intriguingly, PSI is limited by the donor side due to dramatically reduced Cytochrome (Cyt) $b_6f$ content. We propose that Cyt $b_6f$ downregulation represents a protective acclimation strategy, safeguarding PSI at the cost of photosynthetic efficiency under prolonged heat exposures.

## Introduction

The global mean temperature in 2024 was 1.5°C above the pre-industrial era (WMO, 2025). Global warming has led to more frequent and severe heatwaves, disrupting ecosystems and threatening agriculture. The rapidly warming climate, particularly the increased frequency and intensity of heatwaves (IPCC, 2021), significantly impacts plant metabolism. Based on different modelling and field-warming experiments, it was estimated that each degree-Celsius increase in global mean temperature would reduce global yields of wheat by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1% (Zhao et al., 2017). Understanding the mechanisms of heat damage on plant metabolism is fundamental for mitigation strategies that prevent the loss of plant productivity under increasing temperatures.

Photosynthesis is considered to be among the most prone cell functions to be damaged upon heat stress (Allakhverdiev et al., 2008). Light-driven photosynthetic electron transfer is conducted by membrane-embedded large protein complexes: photosystem (PS) II and PSI, which contain peripheral light harvesting antennae (LHCII and LHCI, respectively), and are interconnected by cytochrome (Cyt) $b_6f$ and two mobile electron carriers: plastoquinone (PQ) and plastocyanin (PC). The electrons, originating from water splitting, are transferred from one complex to another and ultimately used for reducing $NADP^+$ to NADPH, while the simultaneously formed proton gradient across the thylakoid membrane is released by ATP synthase (ATPase) for the production of ATP. Generated NADPH and ATP are mainly used in assimilation of atmospheric $CO_2$ by Calvin-Benson-Basham (CBB) cycle. Balancing the energy supply from electron transfer reactions with the downstream metabolic demands is essential for efficient photosynthesis under fluctuating environmental conditions. To cope with fluctuations in abiotic stresses, plants have developed mechanisms to regulate their photosynthetic apparatus according to environmental cues (Rochaix, 2014). These mechanisms involve adjustments in the light harvesting antennae to balance the amount of excitation energy between PSII and PSI

(state transitions), dissipation of excess absorbed energy as heat (NPQ) and regulation of the electron transfer chain at Cyt $b_{6f}$ (photosynthetic control) and at PSI (cyclic electron transfer).

Excessive heat affects all aspects of plant photosynthesis from electron transport to carbon metabolism (Berry and Björkman, 1980; Inoué et al., 1987; Sharkey, 2005; Sharkey and Zhang, 2010). Carbon assimilation, particularly, is reduced due to impaired activity of Rubisco activase (RCA) (Feller et al., 1998; Law and Crafts-Brandner, 1999; Crafts-Brandner and Salvucci, 2000; Rokka et al., 2001). The effects of high temperature stress on light reactions are less understood, and notably, many of the existing studies are based on acute single exposure *in vitro* experiments (Gounaris et al., 1983; 1984; Sundby et al., 1986; Inoué et al., 1987; Enami et al., 1994; Semenova, 2004 Komayama et al., 2007), and thus there is limited knowledge on *in vivo* effects of long-term repeated heat exposures on photosynthetic light reactions. The reported effects of high temperature on light reactions include dissociation of the peripheral LHCII antenna from PSII core (Armond et al., 1980; Sundby et al. 1986; Srivastava et al., 1997; Mathur et al., 2011), destacking of thylakoid membranes (Gounaris et al., 1983; 1984), disruption of PQ-pool (Pshybytko et al., 2008) and of oxygen evolving complex (OEC) (Santarius, 1975; Enami et al., 1994). Indeed, PSII has long been considered to be the most prone ETC component to high temperature stress (Berry and Björkman, 1980), in comparison with the thermostable PSI (Ivanov et al., 2017).

To address this knowledge gap, we studied the effects of physiologically relevant recurring heat stress on photosynthetic electron transfer reactions and photosynthetic machinery. *Arabidopsis thaliana* plants were subjected daily to 38°C for four hours during their whole life cycle. In this setup, plants begin to acclimate to long-term recurring elevation in temperature, but also experience short-term stress due to daily heat periods. The effects of daily heat exposure on plant growth, electron transfer reactions and photosynthetic proteins and protein complexes were studied using chlorophyll-fluorescence and absorbance-based methods, immunoprobing and native-PAGE. We show that under these conditions both photosystems remain structurally intact, but their photochemistry is impaired. Intriguingly, we show that daily heat stress results in dramatic downregulation of the Cyt $b_6f$ complex and thus donor-side limitation of PSI. We propose that in heat-acclimated plants this adjustment acts as protective mechanisms, restricting electron flow to protect PSI complex, with a concomitant reduction of photosynthetic activity.

## Results

### *Long-term daily heat exposure results in stunted growth and reduced content of photosynthetic pigments*

To study the effects of daily heat exposures on photosynthesis, plants were grown in chamber where temperature was elevated daily for four hours from 23 °C to 38°C throughout their lifecycle. Control (Ctrl) plants were grown at constant 23 °C. The plants were photographed weekly from week one to five (Fig. 1a) and the biophysical and biochemical characterization was conducted on plants at the end of the growth period,

at week five. As demonstrated in Fig. 1a (lower panel), daily heat treatment resulted in stunted growth, fresh weight of the plants reaching only ~40% of that of Ctrl plants (Fig. 1b), as well as altered rosette morphology with elongated petioles and leaves elevated from the soil. Further, strong hyponastic leaf movement was observed during the heat exposures: the petioles started to bend upwards from the soil during the high temperature period (Supplementary video). Further, in heat-acclimated plants, the accumulation of chlorophyll (Chl) *a* and *b* was reduced by ~30% with no significant effects on Chl *a/b* ratio, as compared to Ctrl (Fig. 1c, 1d). Moreover, the accumulation of carotenoids (xantophylls and carotenes) was reduced by ~30% (Supplementary Fig. 1).

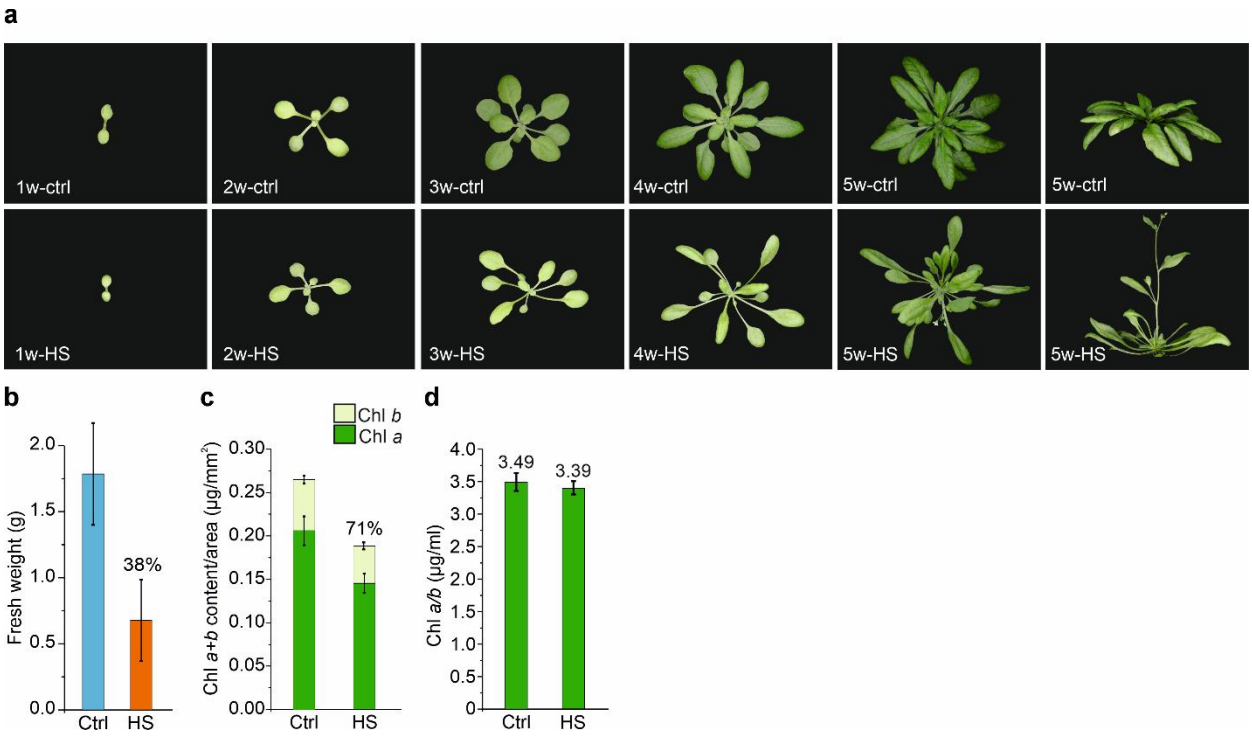


**Figure 1. The effect of prolonged daily heat exposure on (a) plant growth phenotype, (b) fresh weight (c, d) chlorophyll content and chlorophyll a/b ratio.** Plants were grown for five weeks in growth chamber where temperature increased daily from 23 °C to 38 °C for four hours (HS), whereas control (Ctrl) plants were grown at constant 23 °C. Fresh weight (n = 15) and chlorophyll (Chl) content (n = 27) were measured at week five.

### *Daily heat stress alters thylakoid ultrastructure and number of grana per chloroplast*

Earlier reports have demonstrated complete linearization of the thylakoid membrane system upon sudden exposure to high temperature (Gounaris et al., 1983; 1984). To study the effects of daily heat exposure on the thylakoid membrane ultrastructure, leaf samples were collected in the middle of the heat period from 5-week-old plants. Chloroplast morphology and thylakoid membrane ultrastructure were studied using transmission electron microscopy (TEM). As demonstrated in Figure 2, no linearization of the thylakoid membrane was observed. Heat-acclimated plants showed higher number of grana per chloroplast (52 ± 2) compared to Ctrl conditions (33 ± 10), and the average height of grana was significantly smaller in heat-acclimated plants (107.6

± 60.4) compared to Ctrl plants (120.4 ± 60.4) (Fig. 2c). Additionally, daily heat exposure resulted in larger plastoglobuli compared to Ctrl conditions (Fig. 2a, b).

**Figure 2. Transmission electron microscopy analysis of chloroplast ultrastructure.** Samples were collected in the middle of light and heat period. Representative micrographs of chloroplasts from a) control (Ctrl) and b) heat (daily 38°C) acclimated (HS) plants. 8000 × magnification was used; 500 nm scale bar is indicated at bottom-right corner. c) Height of individual grana stacks were measured from 202 (ctrl) and 194 (HS) grana stacks. Mann-Whitney Rank Sum Test showed statistical significance between Ctrl and HS plant grana height (p = 0,014).

### *PSII and PSI are structurally intact but functionally impaired under prolonged heat stress*

Previous studies have demonstrated that PSII-LHCII complex is the most prone ETC component to high temperature-induced damage, resulting in LHCII disconnection from PSII (Armond et al., 1978; 1980), degradation of the PSII core protein D1 as well as disassembly of the OEC complex (Enami et al., 1994; Yamashita et al 2008). To investigate the effects of daily heat exposures on PSII-LHCII supercomplexes, thylakoid membranes from heat-acclimated and Ctrl plants were isolated and solubilized with non-ionic detergent β-dodecyl maltoside (β-DDM), followed by separation with Blue Native (BN)-PAGE. In contrast to earlier studies, heat-acclimated plants showed enhanced association of LHCII to PSII core. Fig. 3a shows high accumulation of large forms of PSII-LHCII supercomplexes (C2S2M2 representing PSII core dimer (C2) attached to two strongly (S2) and one (M) or two (M2) moderately bound LHCII trimers). Moreover, heat-acclimated plants accumulate reduced levels of detached M-LHCII complexes and PSII monomers (Fig. 3a). However, no differences were observed in the overall abundance of the PSII reaction center protein D1 or the LHCII protein Lhcb1 between heat-acclimated and Ctrl plants (Fig. 3b). Notably, the PSII core protein CP43 as well as LHCII proteins were less phosphorylated in heat-acclimated plants than in Ctrl (Fig 3b).

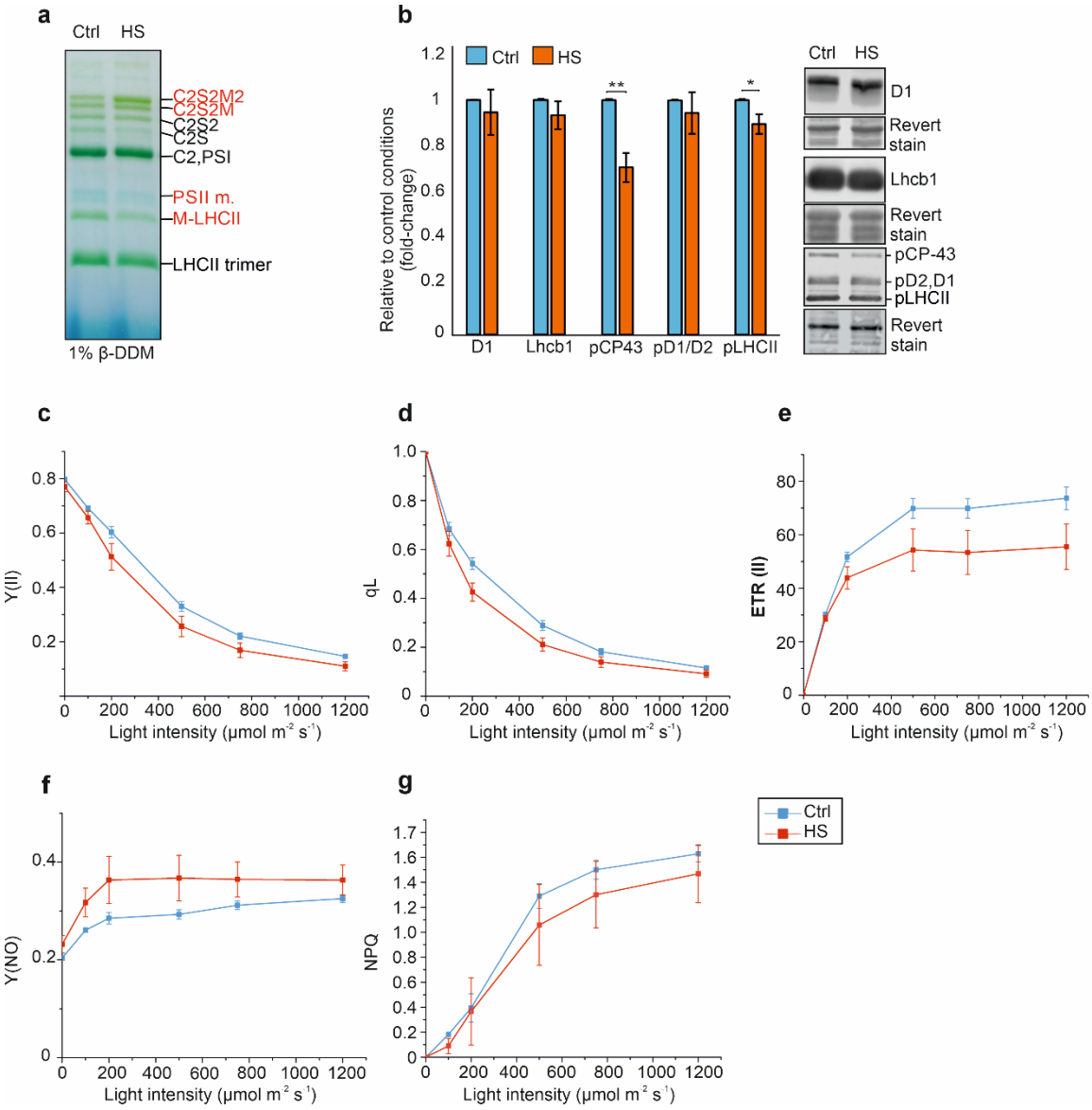


**Figure 3**. **The effects of daily heat stress (HS) on PSII-LHCII.** a) BN-PAGE for thylakoids isolated from control (Ctrl) and HS plants using β-dodecyl maltoside (β-DDM) for solubilization. 5 µg of Chl was loaded per lane. Complexes showing differences between Ctrl and HS are marked in red. b) Western blots and protein quantification from thylakoid membrane proteins isolated from Ctrl and HS plants using antibodies against D1, Lhcb1 and Phosphorylated Thr/Ser. Quantifications are normalized to Ctrl. Statistical significance according to student's t-test is indicated with an asterisk (* = < 0.05, ** = < 0.01) (n = 3 biological replicates). c) – d) Light response curves were recorded using chlorophyll *a* fluorescence with Dual KLAS-NIR system. Quantum yield of PSII (Y(II)) (c), proportion of open PSII reaction centers (qL) (d), PSII electron transport rate (ETR) (e), non-regulated non-photochemical quenching (NPQ) (f) and NPQ (g). n = 6 individual plants.

Although heat-acclimated plants accumulated structurally intact C2S2M2 supercomplexes, quantum yield of PSII (Y(II)), electron transfer rate through PSII (ETR (II)) and the proportion of open PSII reaction centers (qL) were lower in heat-acclimated plants as compared to Ctrl (Fig. 3c-e). Since no differences were observed in the abundance of PSII core proteins or physical disconnection of the LHCII antenna from the PSII core (Fig 3a, b), it seems that a fraction of PSII-LHCII complexes is inactive, but not degraded. Although NPQ was not markedly affected in heat-acclimated plants (Fig. 3g), daily heat exposure resulted in high Y(NO) at all light intensities, reflecting the amount of energy that is dissipated as heat and fluorescence via non-regulated

134 quenching (Fig. 3f). This suggests that the absorbed energy is not properly used for photochemistry, but rather
135 wasted in uncontrolled ways.

136 PSI is considered to be relatively stable under acute heat stress (Ivanov et al., 2017). Previous studies have
137 demonstrated enhanced LHCII phosphorylation and increased absorbance cross-section of PSI in darkness
138 (Ivanov and Velitchkova, 1990; Mohanty et al., 2002; Nellaepalli et al., 2011). To study PSI composition,
139 thylakoid membranes isolated from heat-acclimated and Ctrl plants, were solubilized with digitonin in ACA
140 buffer, which preserves the labile interaction of LHCII with PSI, while solubilizing complexes from the grana
141 stacks, grana margins and stroma lamellae (Rantala et al., 2017). As demonstrated in Figure 4a, the
142 accumulation of PSI complexes did not differ between Ctrl and heat-acclimated plants. Since PSI complex co-
143 migrates with PSII dimer in BN-PAGE, we further studied the PSI accumulation with Western blot analysis,
144 which showed even higher abundance (~20%) of PSI reaction center subunit PsaB in the heat-acclimated plants
145 (Fig. 4b). P700 redox changes, recorded by Dual/KLAS-NIR spectrophotometer (DKN), revealed that
146 although there were no defects in the PSI accumulation, the PSI quantum yield (Y(I)) and electron flow through
147 PSI (ETR(I)) were lower in heat-acclimated plants at nearly all light intensities (Fig. 4c, d). Moreover, in line
148 with the reduced LHCII phosphorylation level (Fig. 3b), heat-acclimated plants exhibited slightly lower
149 accumulation of the state transition -specific PSI-LHCII (Fig. 4a). To study the relative excitation of PSII and
150 PSI, low temperature fluorescence spectra was recorded. Interestingly, increased F735(PSI)/F685(PSII) ratio
151 was observed in heat acclimated plants (Fig. 4e), indicating higher relative excitation of PSI.

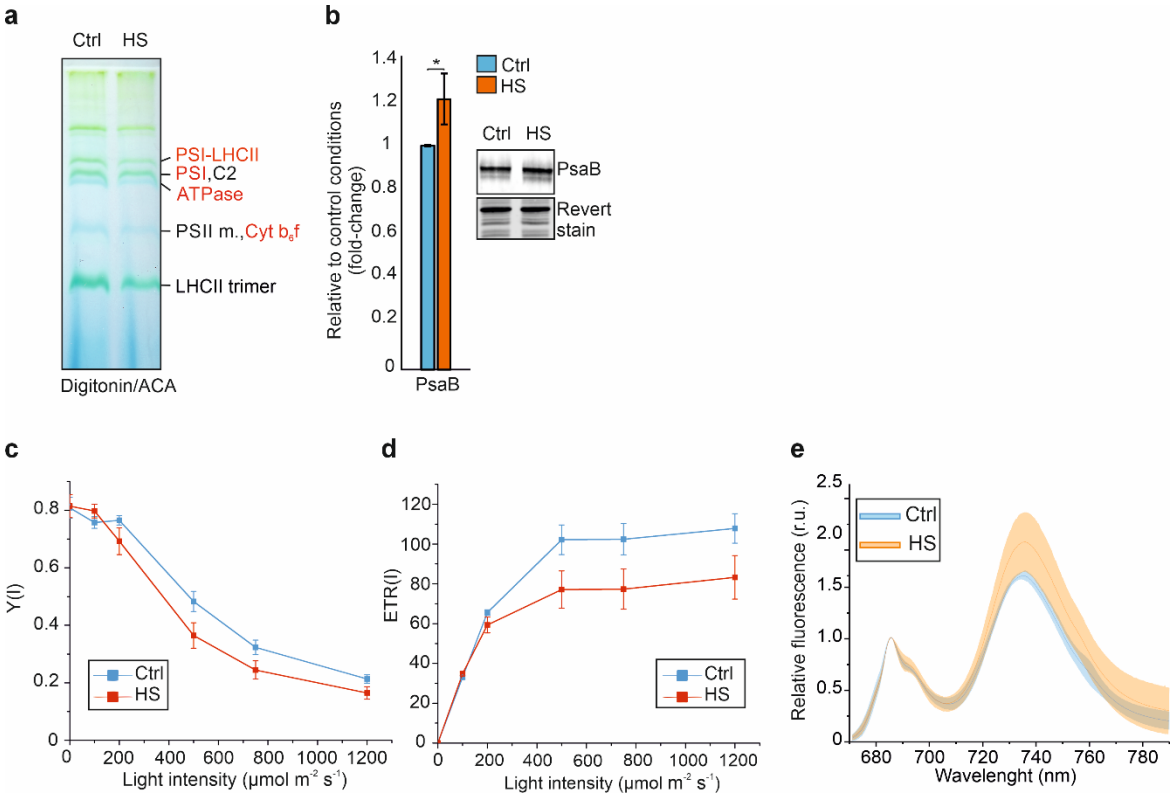


**Figure 4. The effects of daily heat stress (HS) on photosystem PSI.** a) BN-PAGE analysis of native protein complexes from thylakoids of control (Ctrl) and heat acclimated (HS) plants using digitonin ACA buffer for solubilization. 5 µg of Chl was loaded per lane. Complexes showing differences between Ctrl and HS are marked in red. b) Western blot analysis and quantification of PsaB protein from thylakoids isolated from Ctrl and HS. The quantifications were normalized to ctrl. Statistical significance according to student's t-test is indicated with an asterisk (* = < 0.05, ** = < 0.01) (n = 3 biological replicates). c) – d) Light response curves were recorded using P700 absorption spectroscopy with Dual KLAS-NIR system to record c) PSI quantum yield (Y(I)), d) and PSI electron transport rate (ETR) (n = 6 individual plants). e) 77K fluorescence spectra from Ctrl and HS thylakoids (n = 3 biological replicates).

### *Downregulation of Cyt $b_6f$ complex limits electron transfer at PSI donor side*

To determine whether the declined PSI photochemistry detected upon daily heat exposures (Fig. 4) resulted from defects in PSI donor or acceptor side, we measured the acceptor and donor side limitations of PSI and the relative redox states of P700, PC and Fd (Fig. 5 a – c). Figure 5a shows that Y(NA) across all light intensities is lower in heat-acclimated plants, indicating no limitations at PSI acceptor side. This result is in line with the less reduced Fd in heat-acclimated plants as compared to Ctrl plants (Fig. 5c). In contrast, Y(ND) was substantially increased in heat-acclimated plants (Fig. 5b). Moreover, both PC and P700 were more oxidized (Fig. 5c), implying that the electron flow from Cyt $b_6f$ to PSI is restricted, resulting in PSI donor side limitation. This is indeed supported by the dramatically reduced Cyt $b_6f$ content in heat-acclimated plants, as shown by BN-PAGE (Fig. 4a). This result was confirmed by 30-40% decreases in CytF and Rieske subunits of the Cyt $b_6f$ complex in Western blot analysis (Fig. 5d). In addition, ATPase complex (Fig. 4a) and the ATPase subunit ATPβ (Fig. 5d) were significantly less abundant in heat-acclimated plants.

In order to analyze the effects of downregulated Cyt $b_6f$ and ATPase on proton motive force (*pmf*), electrochromic shift (ECS) from dark to light transition was measured with a JTS-150 spectrometer. Light-

induced *pmf* was determined from the dark interval relaxation kinetics of the ECS signal (Fig. 5e). Surprisingly, heat-acclimated plants showed similar *pmf* as the Ctrl plants, remaining even higher from 50 s onwards. To further inspect the difference in the later measuring points, thylakoid membrane conductivity ($gH^+$) (Fig. 5f) and proton flux to the lumen ($vH^+$) (Fig. 5g) were calculated from ECS measurements. No major differences were detected in $gH^+$, despite the ATPase downregulation. $vH^+$ was higher at measuring point 60 s, which might explain the higher *pmf* in heat-acclimated plants, and possibly indicates enhanced CET around PSI. Based on these results, it seems that at growth light conditions heat-acclimated plants have no impairment in generating pH gradient despite dramatically lower Cyt $b_6f$ levels.

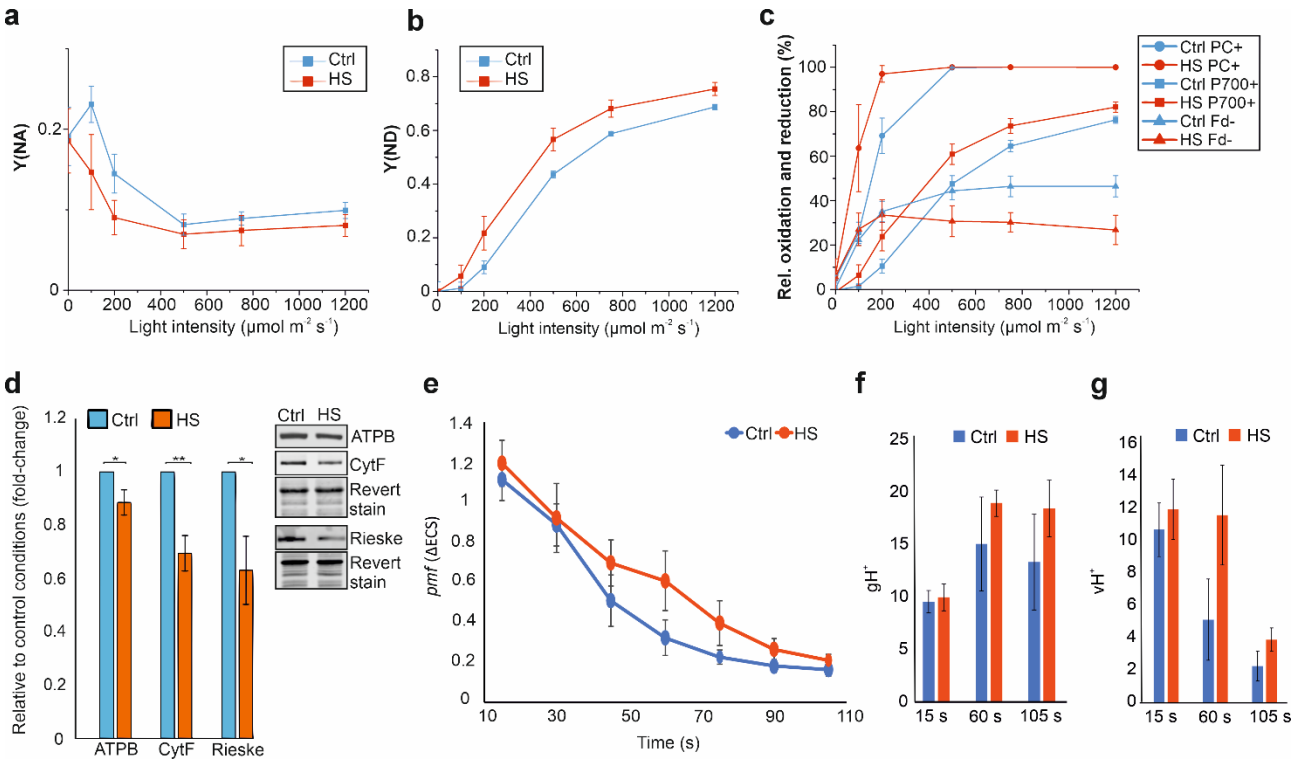


**Figure 5. Assessing the effect of daily heat stress (HS) on donor and acceptor side limitation of PSI and proton motive force (*pmf*).** Dual KLAS-NIR was used to measure a) PSI acceptor side limitation (Y(NA), b) donor side limitation (Y(ND) and, c) relative oxidation/reduction of plastocyanin (PC), P700 and ferredoxin (Fd). (n = 6 individual plants). d) Western blots and protein quantifications from thylakoids of Ctrl and HS plants using antibodies against ATPB, Cyt F and Rieske. Statistical significance according to student's t-test is indicated with an asterisk (* = < 0.05, ** = < 0.01) (n = 3 biological replicates). e) *pmf* was measured during transitions from dark to 120 µmol photons m−2 s−1 of green actinic light. Absorbance difference between 500 and 480 nm (electrochromic shift, ECS) was measured with a JTS-150 spectrometer, and light-induced *pmf* was determined from the dark interval relaxation kinetics of the ECS signal. Eight individual plants were measured/condition. f) Conductivity of the thylakoid membrane ($gH^+$) after 15, 60 and 105 sec of illumination as in e). $gH^+$ was determined as the inverse of the time constant of first-order post-illumination decay kinetics of the ECS signal. g) Thylakoid proton flux ($vH^+$), calculated as pmf × $gH^+$ from same data points as in f).

## Discussion

Rapid increase in ambient temperature affects multiple aspects of plant growth, especially photosynthetic reactions (Berry and Björkman, 1980). Currently, holistic understanding of the effects of physiologically relevant heat stress on the thylakoid membrane reactions is completely lacking. Long-term changes in environmental conditions trigger changes in the metabolic consumption of ATP and NADPH, and the

198 stoichiometry of photosynthetic complexes is dynamically regulated to meet the metabolic needs (Schöttler
199 and Tóth, 2014). It has been suggested that these dynamic changes are mediated by the redox state of
200 photosynthetic electron transfer chain, i.e. PQ pool and Cyt $b_6f$, which act as a signaling network to regulate
201 gene expression levels in nucleus and chloroplast (Anderson et al., 1997; Pfannschmidt, 2003).

202 Long-term daily heat stress dramatically declines Chl content (Fig. 1d), which is in line with earlier reports
203 (Fatma et al., 2021; Mustafa et al., 2021) and is attributed to increased accumulation of chlorophyllase and
204 Chl-degrading peroxidase (Rossi et al., 2017). Interestingly, the abundancies of Chl-binding PSII-LHCII and
205 PSI are not reduced (Fig. 3a, b; 4a, b), and thus it seems that fewer Chls are associated with the complexes
206 under prolonged recurring heat stress. In heat-acclimated plants, the overall abundance of D1 and Lhcb1
207 remain unaltered and strikingly, the C2S2M2 and C2S2M supercomplexes are the dominant forms of PII-
208 LHCII, indicating stable association of LHCII with the structurally intact PSII core. This is in contrast with
209 earlier reports demonstrating D1 degradation (Komayama et al., 2007; Yamashita et al., 2008) and physical
210 disconnection of the LHCII antenna from the PSII core (Armond et al., 1980; Sundby et al. 1986; Srivastava
211 et al., 1997; Mathur et al., 2011). Lower fraction of the captured light energy is utilized in photochemistry, and
212 correspondingly higher fraction is passively dissipated due to closed reaction centers under elevated
213 temperature (Fig. 3a-d). The accumulation of non-functional PSII-LHCII complexes in the heat-acclimated
214 plants could indicate impairment in the PSII repair cycle. During the PSII repair cycle, phosphorylated PSII
215 dimer monomerizes and the monomers migrate to non-appressed thylakoid membranes. The PSII core proteins
216 D1, D2 and CP43 are dephosphorylated (Koivuniemi et al., 1995; Rintamäki et al., 1996; Rokka et al., 2000)
217 before proteolytic degradation of the D1 protein. Newly synthesized D1 protein is inserted to PSII subcomplex
218 and the new functional complex is assembled. In heat-acclimated plants, the PSII repair cycle intermediate,
219 PSII monomer (Aro et al 2005), is almost completely absent (Fig. 3a), which supports the hypothesis of
220 impaired PSII repair cycle. Since we did not observe deficiencies in the core protein dephosphorylation (Fig.
221 3b), it seems unlikely that the (de)phosphorylation of the core proteins would explain the defects in the repair
222 cycle.

223 We show that daily heat stress does not affect the accumulation of the PSI complex and, in fact, increases the
224 PSI reaction center protein PsaB content (Fig. 4a, b). However, association of PSI with LHCII antenna trimer
225 is reduced (Fig. 4a), yet more excitation energy is directed to PSI (Fig. 4e) in heat-acclimated plants. Such
226 increase of relative PSI excitation could indicate randomization of the thylakoid protein complexes and
227 consequent energy spill-over from PSII to PSI (Barber, 1980; Ivanov and Velitchkova, 1990). Our analysis of
228 the thylakoid ultrastructure shows smaller grana stacks (height) in heat-acclimated plants, but not full
229 linearization of the thylakoid membrane (Fig. 2) and therefore unlikely randomization of PSII and PSI. More
230 detailed analysis of the protein complex distribution in heat-acclimated plants is needed to understand the
231 lateral heterogeneity of the complexes under heat stress conditions and the possible excitation energy spill-
232 over from PSII to PSI.

233 Electron flow beyond $Q_A$ is suppressed by dramatic Cyt $b_6f$ downregulation, which restricts electron flow to
234 PSI ensuring more oxidized P700. Decrease in the Cyt $b_6f$ content likely serves to protect PSI from excessive
235 reduction and ROS generation. Indeed, Cyt $b_6f$ function has been shown to be essential for protection of PSI
236 (Joliot and Johnson, 2011), since its dysregulation show extensive PSI photoinhibition. Protection of PSI is
237 crucial, as it lacks similar repair machinery as PSII: once PSI is damaged, the repair process requires synthesis
238 and assembly of new complex (Sonoike, 2011). Despite the lower Cyt $b_6f$ content, we did not observe
239 difference in the *pmf* between the heat-acclimated and ctrl plants (Fig. 5e). Intriguingly, proton flux to the
240 lumen was higher in heat-acclimated plants (Fig. 5g), which might indicate increased CET during heat period.
241 Indeed, previous studies have reported activation of CET under high temperature (Bukhov et al., 1999;
242 Schrader et al., 2004; Zhang and Sharkey, 2009), which has been hypothesized to maintain ATP content upon
243 increased thylakoid leakiness induced by heat. Further ECS measurements after the daily heat period are
244 required to distinguish short-term mechanisms induced by heat period from the acclimatory responses.

245 The limitation of photosynthesis under acute single heat exposures most likely differs from long-term repeated
246 exposures to 38 °C that we used in our experimental setup, since our results seem to be controversial to many
247 previously reported results. Moreover, our results demonstrate acclimatory responses of the photosynthetic
248 apparatus in addition to short-term regulation. Taken together, our results demonstrate that daily heat stress
249 impairs function of PSII and PSI, although both remain structurally intact. We propose that non-functional
250 PSII-LHCII complexes accumulate due to defects in PSII repair cycle. PSI is limited at the donor side, and
251 downregulation of Cyt $b_6f$ possibly serves as an acclimatory strategy to protect PSI under repeated heat
252 exposures (Fig 6).

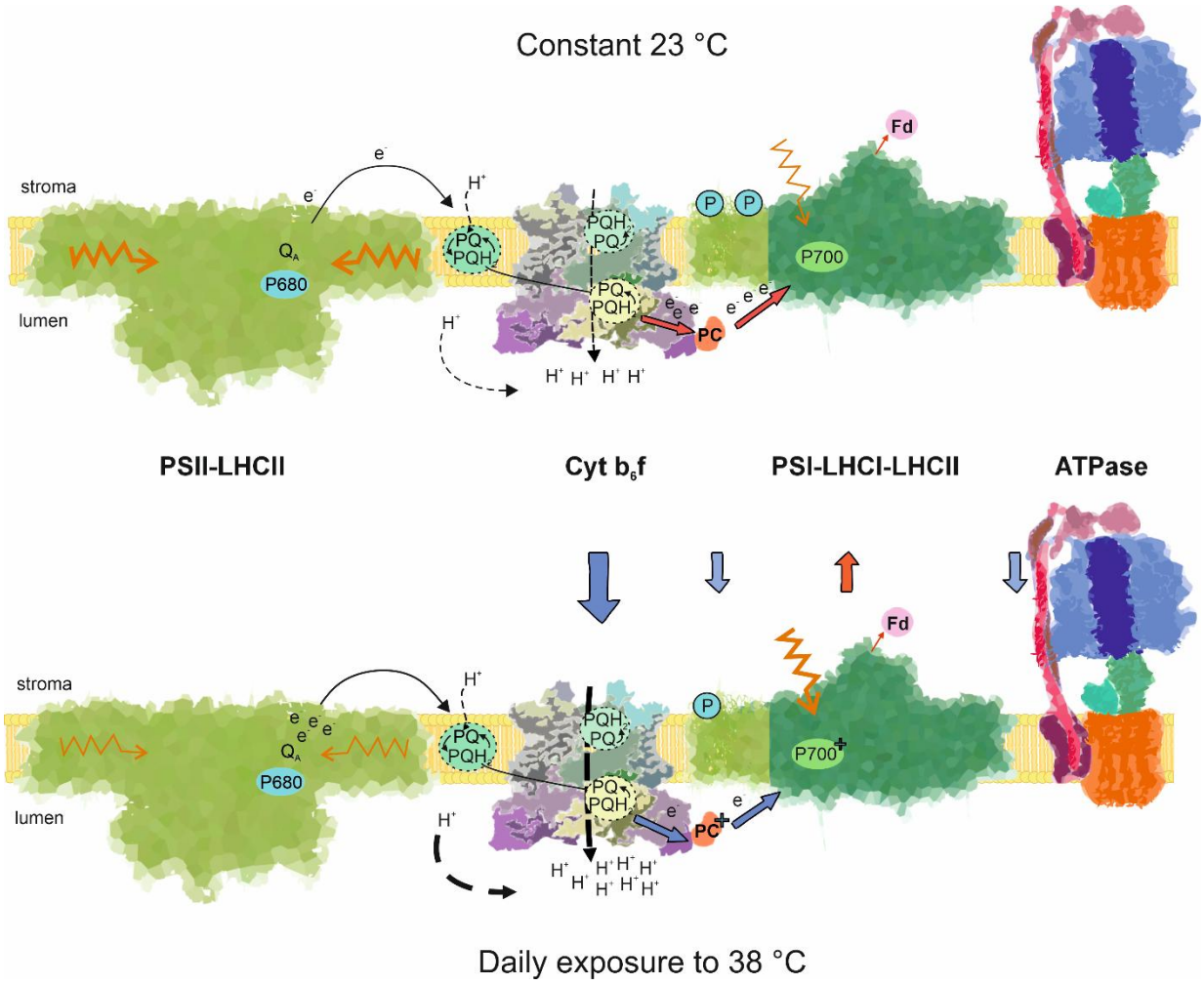


**Figure 6. Summary of the effects of prolonged daily heat exposure to photosynthetic light reactions.** In contrast to control conditions (constant 23 °C), plants grown under conditions where temperature elevated daily to 38 °C for four hours show differences in their photosynthetic light reactions. Blue arrows represent downregulated (Cyt $b_6f$, ATPase and state transition specific PSI-LHCI-LHCII complex) and red arrow upregulated (PSI core subunit PsaB) protein complexes. Heat-acclimated plants show reduced PSII photochemistry despite containing intact supercomplexes. Additionally, the relative PSI excitation is higher in heat-acclimated plants (indicated with orange arrows) despite lower accumulation of PSI-LHCI-LHCII complex. Downregulation of Cyt $b_6f$ decreases electron flux to PSI, leading to more oxidized plastocyanin (PC) and P700. Intriguingly, daily heat period result in increased proton influx lumen, that possibly indicates increased cyclic electron transfer around PSI.

## Materials and methods

### *Plant growth conditions and phenotyping*

*Arabidopsis thaliana* ecotype Columbia-0 (Wt) was grown under 12 h light/ 12 h dark cycle at photosynthetic photon flux density (PPFD) of 130 µmol $m^{-2}$ $s^{-1}$ (VALOYA LUMI-CS LL120 LED), + 23°C and relative humidity of 50% for 4-5 weeks. Heat-acclimated plants were grown in a chamber where temperature elevated daily from 23 to 38°C for four hours and RH was 50-70% depending on the temperature. Light intensity and cycle were same as in Ctrl.

The phenotypes of heat-acclimated and Ctrl plants were imaged weekly throughout the growth period. The images were processed with Adobe Photoshop (version 26.6.1, Adobe Systems Inc.).

271 ***Pigment analysis (DMF)***

272 Leaf discs (63.6 $mm^2$) collected from 5-week-old Wt plants grown under daily heat stress and Ctrl conditions
273 were immersed in *N,N*-dimethylformamide (DMF), then incubated overnight in darkness. Leaf debris was
274 pelleted by centrifugation (5 min, 20 000 x g, RT), and absorbances of the supernatants were measured at
275 663.8, 646.8, and 480 nm with spectrophotometer (UV-1800, Shimadzu). Pigment concentrations were
276 calculated according to Wellburn (1994) using the following equations: Chl *a* = 12A663.8 - 3.11A646.8, Chl
277 *b* = 20.78A646.8 – 4.88A663.8, Car = (1000A480 – 1.12Chl *a* – 34.07Chl *b*)/245. Nine leaf discs from three
278 biological replicates were measured (n=27).

279 ***Transmission electron microscopy (TEM)***

280 Leaf samples for TEM were prepared according to Hyman & Jarvis (2011). Briefly, the leaf fragments (1 x 1
281 x 5 mm) from 5-week-old wild-type *Arabidopsis thaliana* plants were cut at 90 ° angle to the midrib from
282 leaves of same developmental stage. The samples were collected in the middle of light and heat period. Ten
283 leaf fragments from three individual plants were dissected in fixative solution (2.5% (w/v) glutaraldehyde, 0.1
284 M sodium phosphate buffer, 4.0% (w/v) paraformaldehyde, pH 7.2) and incubated in the fixative for four hours
285 at room temperature. Samples were then transferred to 4 °C and stored overnight. The sample preparation for
286 TEM was done according to Hyman & Jarvis (2011). Samples were analyzed with JEM-1400 Plus
287 Transmission Electron Microscope.

288 ***Thylakoid isolation***

289 Thylakoid proteins were isolated according to Järvi et al., 2011. Chl concentration was determined from the
290 samples according to Porra et al., 1989. Isolations were done at the middle of heat period.

291 ***Polyacrylamide gel electrophoresis (PAGE)***

292 For SDS-PAGE, thylakoid proteins were solubilized with 2× Laemmli buffer (Laemmli, 1970) supplemented
293 with 6 M urea and run on 12% acrylamide gels. The proteins were electroblotted onto Immobilon-FL
294 membrane (Merck Millipore) with Trans-blot® (Bio-Rad) device, blocked with 5% milk or bovine serum
295 albumin (BSA) in TTBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and incubated with
296 primary antibody overnight. The sample was loaded on Chl basis as follows: 0.5 µg of Chl for Phospho-Thr/Ser
297 antibody (Cell Signaling Technology, 9381S; 1:6000) and 1 µg of Chl for D1 (DE-loop antibody (Kettunen et
298 al., 1996); 1:8000), PSAB (AS10695; 1:2500, Agrisera), ATPB (AS05085; 1:5000, Agrisera), Cyt f (AS06119;
299 1:1000, Agrisera), Rieske (AS08330; 1:5000) and Lhcb1 (AS01004; 1:2000). The membrane was incubated
300 with a secondary antibody IRDye® 800CW Goat anti-Rabbit IgG (1:20 000 in 1% milk/TTBS) and scanned
301 with Li-Cor Odyssey CLx.

302 For BN-PAGE, gels and samples were prepared as in Rantala et al., 2018. Thylakoid samples were solubilized
303 with 1% digitonin or β-DMM, and samples containing 5 µg of Chl were separated on BN gels.

304 ***Chlorophyll a fluorescence and P700 absorbance spectroscopy***

305 DKN spectrophotometer (Heinz Walz GmbH) was used to measure Chl *a* fluorescence from PSII with pulse-
306 modulated 540 nm measuring light, and redox changes of P700, PC and Fd were measured by monitoring
307 absorbance changes at 780–820, 820–870, 840–965 and 870–965 nm. PC, P700 and Fd redox changes were
308 deconvoluted based on differential model plots (DMPs) (Klughammer and Schreiber, 2016). Normalization of
309 PC, P700 and Fd redox changes was done according to the maximal redox changes that were determined with
310 the software's NIR Max-script. Steady state oxidation and reduction levels were calculated as relative $PC_{ox}$,
311 $P700_{ox}$ and $Fd_{red}$. Light response curves were measured by illuminating leaves with 635 nm red actinic light
312 for 5 min with the following light intensities: 100, 200, 500, 750 and 1200 µmol $m^{-2}$ $s^{-1}$. Plants were dark-
313 adapted for 1 h prior measurement, and measurements on heat-acclimated plants were done at the middle of
314 heat treatment period.

315 ***ECS measurements***

316 The ECS signal arising from light-induced absorbance difference between 550 nm and 515 nm were recorded
317 with JTS-150 spectrophotometer (BioLogic, Seyssinet-Pariset, France). ECST, maximum amplitude of the
318 ECS, was calculated as the difference between total ECS in light and an $Y_0$ value obtained from the first-order
319 exponential fit to the decay kinetics of the ECS signal during a dark interval. Dark intervals were given every
320 15 s. The $gH^+$ parameter (thylakoid membrane conductivity to protons) was calculated as the inverse of the
321 time constant of a first-order exponential fit to ECS decay kinetics during a dark interval (Avenson et al., 2005;
322 Cruz, 2005). The $vH^+$ parameter (the rate of proton flux over the thylakoid membrane) corresponding to the
323 initial slope of the decay of the ECS signal upon cessation of illumination was calculated as *pmf* x $gH^+$ (Cruz,
324 2005). Plants were dark-adapted for 1 h prior measurement and measurements on heat-acclimated plants were
325 done at the middle of heat treatment period.

326 ***Funding***

327 This work was supported by the Research Council of Finland grant 355787 (L.L., T.T., L.V., M.R. and P.M.),
328 the Finnish Cultural Foundation (00220578 Keskusrahasto for L.L., 00230972 for M.R.) and Climate chamber
329 was built with funding from BioCity Turku.

330 ***Author Contributions***

M.R. and L.L. planned the experiments and wrote the manuscript with contributions from P.M.; L.L., M.R., T.T. and L.V. performed the experiments. All contributed to the data analysis and participated in the writing of the final manuscript.

***Acknowledgements***

We thank Professor Eva-Mari Aro for valuable advice on TEM sample preparation and for expert guidance with the imaging. We thank Dr. Lauri Nikkanen for his advices with DKN and for JTS operation and data handling. Dr. Steffen Grebe, Dr. Laura Wey, Dr. Tapio Lempiäinen and Dr. Minna Konert are thanked for discussions and valuable insights. Versa Elements is thanked for building the heat chamber and Mika Keränen, Tapio Ronkainen and Samuli Pyytövaara are thanked for the technical assistance with the growth chamber. TEM specimen preparation and imaging was done at Laboratory of Electron Microscopy, the Institute of Biomedicine, University of Turku. Special thanks to Jenni Laine and Markus Peurla in technical guidance at TEM Laboratory.

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