



**HAL**  
open science

## **Thylakoid grana stacking revealed by multiplex genome editing of LHCII encoding genes**

Zeno Guardini, Rodrigo Gomez, Roberto Caferri, Johannes Stuttmann, Luca Dall'osto, Roberto Bassi

### ► **To cite this version:**

Zeno Guardini, Rodrigo Gomez, Roberto Caferri, Johannes Stuttmann, Luca Dall'osto, et al.. Thylakoid grana stacking revealed by multiplex genome editing of LHCII encoding genes. 2024. <hal-04850139>

**HAL Id: hal-04850139**

**<https://hal.science/hal-04850139v1>**

Preprint submitted on 19 Dec 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

# Thylakoid grana stacking revealed by multiplex genome editing of LHCII encoding genes

Zeno Guardini<sup>1</sup>, Rodrigo L. Gomez<sup>1</sup>, Roberto Caferri<sup>1</sup>, Johannes Stuttmann<sup>2</sup>, Luca Dall'Osto<sup>1</sup> and Roberto Bassi<sup>1,\*</sup>

<sup>1</sup>Dipartimento di Biotecnologie, Università di Verona, Strada Le Grazie 15, 37134, Verona, Italy

<sup>2</sup>Institute for Biology, Department of Plant Genetics, Martin Luther University Halle-Wittenberg, Weinbergweg 10, 06120 Halle (Saale), Germany

\*Corrispondence: roberto.bassi@univr.it

keywords: chloroplast, thylakoids, grana, stroma membranes, stacking, LHC, LHCII, monomeric antennae, photosystem, photoprotection.

## Summary

Land plant chloroplasts differ from algal ones for their thylakoid membranes being organized in grana: piles of vesicles paired by their stromal surface, forming domains including Photosystem (PS) II and its antenna while excluding PS I and ATPase to stroma membranes, connecting grana stacks. The molecular basis of grana stacking remain unclear. We obtained genotypes lacking the trimeric antenna complex (Lhcb1-2-3), the monomeric Lhcb4-5-6, or both. Full deletion caused loss of grana, while either monomers or trimers support 50% stacking. The expression of Lhcb5 alone restored stacking at 50%, while Lhcb2 alone produced huge grana which broke down upon light exposure. Cyclic electron transport was maintained in the lack of stacking, while excitation energy balance between photosystems and the repair efficiency of damaged Photosystem II were affected. We conclude that grana evolved for need of regulating energy balance between photosystems under terrestrial canopy involving rapid changes in photon spectral distribution.

## Introduction

The land plant cell chloroplast contains chlorophylls (Chl) and carotenoids (Car) within its inner membrane system called thylakoids, as recognized already by Meyer in 1883 (Gunning et al., 2006). Its most distinctive feature is the thylakoid organization into grana stacks (Wietrzynski et al., 2020), a feature of land plant species, with size dependent on growth light intensity (Heitz, 1936) and spectra (Iwai et al., 2018; Kyle et al., 1983), which is absent in algae (Vallon et al., 1991). Grana are stacks of round, flattened vesicles (Granick & Porter, 1947) connected to each other by pairs of unstacked membranes, called stromal membranes, intersecting grana at an angle, suggesting a helical arrangement (Paolillo & Reighard, 2011). Freeze-etching

31 electron microscopy (EM) coupled to mutant analysis showed that the photosynthetic pigment-protein  
32 complexes of thylakoid membranes are compartmented: photosystem (PS) II particles in the appressed  
33 region of the grana (Armond & Arntzen, 1977; Simpson & Robinson, 1984); PSI and ATPase complexes hosted  
34 in stromal membranes (Armond et al., 1977; Simpson, 1982). Cytochrome (Cyt) *b<sub>6</sub>f* complex was enriched in  
35 stromal domains and grana margins (Allred & Staehelin, 1985; Olive et al., 1986; Vallon et al., 1991).  
36 Biochemical and immunocytochemical analysis supported differential compartmentation of PSI and PSII  
37 (Anderson & Melis, 1983; Vallon et al., 1991), arguably for preventing excitation energy spillover from PSII to  
38 PSI, owing to the low energy chlorophyll spectral forms of plant PSI-LHCI (Murata, 1969). Since  
39 photosynthetic electron transport occurs linearly between PSs, the Plastoquinone (PQ) diffusion towards Cyt  
40 *b<sub>6</sub>f* is restricted by PSII crowding in grana partitions (Laverne & Joliot, 1991) similar to the diffusion of  
41 plastocyanin (PC) in the narrow thylakoid lumen (Kirchhoff et al., 2011), thus limiting linear electron flow  
42 (LET) rate. Restriction of diffusion also applies to PSII damaged by photoinhibition, which need to reach the  
43 stromal domains in order to be repaired (Mattoo et al., 1989). Thus, the size of granal vs. stromal domains  
44 appear to compromise between the positive effect of ensuring adequate excitation energy to both PSs and  
45 the negative effect of restricting PQ diffusion and PSII repair rate. This is consistent with PQ overreduction  
46 activating STN-7 and -8 kinases, which phosphorylates, respectively, the Lhcb2 subunit trimeric antenna  
47 (LHCII) and PSII core, yielding into reduced granal diameter (Bassi et al., 1989; Hepworth et al., 2021). Thus,  
48 LHCII subunits, diffuse to PSI-LHCI. This boosts PSI photon cross section causing oxidation of plastoquinol  
49 (PQH<sub>2</sub>) (Kyle et al., 1983) and kinase inactivation, balancing PSI vs. PSII activity. It was proposed that the  
50 reduction of grana domains enhances the fraction of Cyt *b<sub>6</sub>f* in stromal membranes and favors cyclic electron  
51 flow (CEF) and ATP synthesis, thus helping to meet the NADPH/ATP ratio required for CO<sub>2</sub> fixation (Kramer &  
52 Evans, 2011) and adding to the regulatory value of grana stacking dynamics. In this context it is important to  
53 clarify the mechanism of grana stacking. Reverse genetics and EM analysis yielded controversial results:  
54 intermittent light grown plants becomes LHCII-deficient and exhibit limited grana (Argyroudi-Akoyunoglou  
55 et al., 1971; Armond et al., 1976); however, *chlorina f2* mutants of barley lack Chl *b* and are depleted in LHCs,  
56 yet they retained extensive grana stacks and photosystem lateral heterogeneity (Bassi et al., 1985; Kim et al.,  
57 2009). Grana stacks were also retained in the *viridis115* barley mutants, lacking PSII core complex, and in the  
58 double mutant *viridis 115 x chlorina f2* (Simpson et al., 1989), suggesting that neither of the two major  
59 thylakoid components located in grana partitions (LHCII and PSII core) was essential for grana stacking.  
60 Chloroplasts lacking grana can be found in bundle sheath (BS) cells of maize leaves, with a reduced PSII  
61 activity and a low LHCII level (Bassi et al., 1995). Also, LHCII of BS exhibited a simpler polypeptide composition  
62 with respect to mesophyll chloroplasts (Bassi & Simpson, 1986) suggesting that specific gene products within  
63 LHC, rather than any member of the family, might be responsible for stacking. Here, we proceeded to the  
64 reverse genetic analysis of grana stacking by a combination of genome editing (GE) and EM. Previous work  
65 showed that PSII core complex-less plants (Belgio et al., 2014; Campoli et al., 2009; Margulies, 1966)

66 enhance stacking, we focused on LHCs: genotypes lacking either monomeric or trimeric LHC gene products  
67 or both were produced and analyzed for stacking by transmission EM. In addition, Lhcb2, a minor component  
68 trimeric LHCII, reported to be needed for state 1 - state 2 transitions (Pietrzykowska et al., 2014), was  
69 expressed as the only LHC component and found to induce the formation of extensive stacking in dark  
70 conditions, which were broken down upon light-induced phosphorylation. Our results show that a major  
71 thylakoid stacking is caused by minority components of the PSII antenna system, namely Lhcb5 (CP26) and  
72 Lhcb2, which respectively mediate the constitutive and dynamic components of thylakoid stacking, which is  
73 required for efficient of PSII repair and balance of excitation energy; instead, it does not significantly change  
74 the rate of linear vs. cyclic electron transport.

## 75 **Results**

### 76 **Construction of *Arabidopsis* genotypes with selectively reduced PSII antenna system by GE**

77 The construction of *Arabidopsis thaliana* genotypes affected in LHC protein composition was performed from  
78 either the knock-out (ko) line *koLhcb3*, missing the Lhcb3 component of the major LHCII complex, or the *NoM*  
79 *koLhcb3* genotype, devoid of the *Lhcb4-6* genes encoding monomeric LHCs: Lhcb4 (CP29), CP26 and Lhcb6  
80 (CP24)(Dall'Osto et al., 2017), as well as Lhcb3 (Supplementary Figure S1-A). To obtain the Lhcb-free  
81 genotype, CRISPR-CAS9 targeted mutagenesis strategy was implemented according to (Ordon et al., 2020):  
82 for each of the 5 LHCBI and 3 LHCBI2 genes, pairs of sg (single guide) RNAs were designed within coding  
83 sequences (Supplementary Table S1), with the aim of producing large DNA deletions, favoring DNA repair by  
84 non-homologous end-joining (NHEJ) (Malzahn et al., 2017). *Lhcb1* and *Lhcb2* genes were separately targeted  
85 to get Lhcb1-less and Lhcb2-less plants, which were then crossed to obtain lines lacking both Lhcb1 and Lhcb2  
86 proteins. Depending on whether this procedure was applied on *koLhcb3* or *NoM koLhcb3* genotype, we  
87 obtained either the *koLHCII* line (lacking all LHCII but retaining monomeric Lhcb4-6 proteins), or the *koLhcb*  
88 line, lacking both the monomeric and trimeric LHCs of PSII. In both cases LHCI proteins were unaffected. The  
89 *low-LHCII* genotype was selected for the level of residual LHCII among the products of incomplete gene  
90 editing of both *Lhcb1* and *Lhcb2*: the two selected lines contain one LHCII trimer per monomeric PSII core  
91 complex. *NoM koLhcb1 koLhcb3* lines (*Lhcb2-only*) retained Lhcb2 as the unique PSII antenna (Supplementary  
92 Figure S1-B, Supplementary Table S2).

### 93 **Growth and pigment composition of LHC-depleted plants**

94 Plants of the different genotypes are shown in Figure 1A upon growth under light-limiting (LL) conditions  
95 ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 8/16h photoperiod, 24 °C). Wild type showed the best growth. *NoM* and the two  
96 *koLHCII* lines accumulated between 45% and 50% with respect to the wild type; while the growth of *koLhcb*  
97 was far more reduced: the two independent lines grew less than 3% with respect to the wild type based on  
98 fresh weight (FW) (Figure 1B). The Chls content per leaf surface displayed a behavior similar to the growth

99 pattern although changes were far smaller: *NoM* showed 25% less Chl vs. the wild type, while *koLhcb* lines  
100 about 50% Chls when compared to *koLHCII* and 25% with respect to the wild type, suggesting that the growth  
101 rate per pigment unit was higher in the presence of monomeric antenna proteins (Figure 1C). The pigment  
102 composition of the different genotypes reflected the abundance of the pigment-protein complexes residual  
103 from gene deletions. Thus, Chl *a/b* ratio was the lowest in *NoM* (3.28 vs 3.51 in the wild type), the highest in  
104 *koLhcb* (6.21) and intermediate in *koLHCII* (5.19), consistent with the lower Chl *b* content of monomeric Lhcb  
105 vs LHCII (Table I). LHCI complexes, unaffected in our genotypes, account for the residual Chl *b* content in  
106 *koLhcb*. The Chl/Car ratio ranged from 3.0 to 3.7 slightly decreasing with LHC content (Table I). The higher  
107 Car /Chl ratio in *koLHC* lines resulted from increased  $\beta$ -carotene accumulation, which doubled in *koLhcb* with  
108 respect to the LHCII-rich wild type and *NoM* (Supplementary Table S3). Neoxanthin decreased by 60% and  
109 80%, respectively in *koLHCII* and *koLhcb*, with a concomitant increase in violaxanthin content owing to the  
110 preferential location of neoxanthin in trimeric LHCII and its absence in PSI-LHCI complexes (Schiphorst &  
111 Bassi, 2020).

112 The major differences in growth between mutants might be due to either the large differences in PSII antenna  
113 size, thus limiting the photon harvesting, and/or to defects in the electron transport chain induced by changes  
114 in the organization of the thylakoid membranes. We thus proceeded to grow plants at higher irradiance,  
115 expected to compensate for antenna size limitation, yet avoiding excess irradiation in order to circumvent  
116 photodamage. Figure 1D shows wild type and LHC mutant genotypes grown at moderately high light (HL, 350  
117  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 8/16h photoperiod, 24°C). *NoM* and *koLHCII* grew similarly while *koLhcb* was far slower  
118 and grew as in control light (150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) (Figure 1B, D). Thus, enhancing irradiance did not  
119 complement for the decreased growth phenotype evidenced under LL conditions and, in some cases, made  
120 it even stronger. This suggests that insufficient photon harvesting was unlikely the major cause of the  
121 differences in growth between genotypes. On the other hand, none of the lines were obviously  
122 photodamaged, or died at 350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , suggesting that photoprotection was strongly reduced.

### 123 **Pigment-protein complexes and photosynthetic function in antenna mutants**

124 The actual pigment-protein content of the genotypes as well as their supramolecular organization was  
125 investigated by non-denaturing Deriphat-PAGE upon solubilization with low (0,8%)  $\alpha$ -DM. Consistent with  
126 previous reports (Dall'Osto et al., 2014, 2017), the *NoM* genotype was depleted in PSII supercomplexes and  
127 over-accumulated the major trimeric antenna LHCII (Figure 2A). The trimeric LHCII band was completely  
128 absent in both *koLHCII* and *koLhcb* genotypes. These were also depleted of high molecular weight (MW)  
129 supercomplexes except for two bands migrating just above PSI-LHCI (BD1 and BD2). A faint green band (BD3)  
130 was detected migrating slightly below trimeric LHCII in both *koLHCII* and *koLhcb*, while a fourth band (BD4)  
131 was detected in both *koLHCII* and *koLhcb*, migrating as the monomeric Lhcb (Figure 2A). Biochemical and  
132 spectroscopical analyses revealed BD1 and BD2 being composed of PSI-LHCI complexes with different LHC

133 complements, BD3 contained dimeric LHCI subunits, BD4 comprised monomeric Lhcb in *koLHCII* and  
134 monomeric LHCI in *koLhcb* (Supplementary Figure S2).

135 The functional antenna size of PSII was determined from the rise time of Chl fluorescence in DCMU-treated  
136 leaves. Compared to the wild type, *NoM* had nearly twice the capacity for PSII photon harvesting, while  
137 *koLHCII* and *koLhcb* antenna size, scored 30% and 45% respectively, vs. the wild type (Figure 2B). When PSII  
138 activity was probed without DCMU in order to determine the maximum quantum yield of photochemistry,  
139 the wild type and *koLHCII* scored very high, i.e. 0.82 and 0.76 respectively. Instead, *NoM* and *koLhcb* showed  
140 a low quantum yield, corresponding to 0.62 and 0.51 respectively (Figure 2C).

#### 141 **Thylakoid membranes organization**

142 Antenna proteins of PSII are the most abundant components of thylakoid membranes, suggesting that  
143 thylakoid organization could be affected in LHC-less genotypes. Indeed, the trimeric LHCII complex has been  
144 suggested to be the major determinant of grana stacking (Standfuss et al., 2005) and of the consequent  
145 domain segregation of the thylakoids, which is typical of land plant chloroplasts and is the basis for multiple  
146 regulation and biogenetic mechanisms essential for photosynthesis in the challenging terrestrial  
147 environment, such as PSII repair cycle and state 1 - state 2 transitions. To verify the effect of LHCII abundance  
148 in the different genotypes, we have analyzed the extent of grana stacks by transmission EM (Figure 3A-G).  
149 All genotypes had chloroplasts with the same size in ultrathin sections (Figure 3H). Grana stacks were defined  
150 as made of at least three thylakoid appressions; the stacks diameter was approx. 450 nm and was found to  
151 be very similar between genotypes, only *NoM* showed a slightly larger grana diameter of approximately 550  
152 nm (Figure 3I). However, see below for the special case of the *Lhcb2-only* genotype.

153 Quantification of grana stack was performed by measuring the surface of grana stack vs. chloroplast area,  
154 since the total length of thylakoid membranes was the same across genotypes, (Figure 3K). Wild type  
155 chloroplasts had about 18% of their section area occupied by grana, a value that was reduced to 11% in  
156 *koLHCII* and *NoM*. Deletion of LHCII in *koLhcb* caused a virtual absence of grana stacks (<1% of stacked area,  
157 Figure 3K), while partial depletion of LHCII in the *lowLHCII* genotype yielded only a very partial recovery on  
158 surface occupied by grana to 3%. We notice that *koLHCII* retains more than 50% of its stacking ability, similar  
159 to *NoM* (Figure 3K), even though these two genotypes display highly diverse content in LHCs: *koLHCII* has  
160 three monomeric LHCs per PSII core, while *NoM* has about 6 LHCII trimers per PSII core, i.e. 60% more LHCII  
161 with respect to the wild type (Dall'Osto et al., 2017). When comparing the stacking efficiency for a single LHC  
162 monomeric unit, the score is 6 times higher than when comparing the wild type to *NoM*, 3 times higher than  
163 comparing *koLHCII* to *lowLHCII*, the latter having the same LHC/PSII core stoichiometry. This suggests that,  
164 although both monomeric and trimeric LHCs contributed to stacking, the monomeric LHCs were most critical

165 determinants for grana formation. In all cases the number of events analyzed was statistically significant  
166 except for the case of *koLhcb*, because most of the chloroplasts did not contain any stacks (Figure 3J).

167 Possibly, one of the clearest effects on grana stacking in previous research was obtained by controlling the  
168 expression of CURT genes (Heinz et al., 2016). Supplementary Figure S3 shows that no major differences were  
169 observed in the abundance of CURT, suggesting LHCII abundance/composition controlled stacking  
170 independently from CURT, or LHCII was needed for CURT activity.

171 To identify whether a specific subunit, among monomeric LHCs, had a special role in stacking, we analyzed  
172 the *ch1* genotype devoid of CAO (Chl *a* oxygenase), which is known for a strongly reduced antenna size (Kim  
173 et al., 2009) caused by Lhcb proteins being destabilized in lack of Chl *b*. CP26, however, is particularly  
174 promiscuous for Chl *a* vs. Chl *b* binding (Croce et al., 2002) and is retained and functional in *ch1* genotype  
175 (Havaux et al., 2007). We have analyzed by EM the extent of grana stacking in *ch1* and *ch1 koLhcb5* mutants  
176 (Figure 3F-G). First, we assessed that the chloroplast area and the diameter of grana in sections were very  
177 similar in *ch1* and *ch1 koLhcb5* genotypes with respect to the wild type and *koLHCII* (Figure 3H,I). *ch1* had  
178 similar stacking as the *koLHCII* genotypes (Figure 3K), suggesting that whatever induced stacking in *koLHCII*  
179 was still present in *ch1*. Instead, stacking was drastically reduced in the *ch1 koLhcb5* genotype (Figure 3G,K),  
180 implying that CP26, among monomeric LHCs, had a prominent function on grana formation.

### 181 **Lhcb2 controls changes in grana stacking during State 1 - State 2 transitions**

182 *Arabidopsis* lines depleted on Lhcb2 proteins lacked state 1 - state 2 transitions (Pietrzykowska et al., 2014).  
183 The GE of *Lhcb1* in the *NoM koLhcb3* background (Supplementary Figure S1) produced a genotype retaining  
184 Lhcb2 as the only PSII antenna (Ordon et al., 2020). Wild type and mutant genotypes analyzed above showed  
185 grana diameters between 400 and 550 nm (Figure 3I), (Kirchhoff, 2019). The *Lhcb2-only* plants were striking  
186 different with respect to the *lowLHCII* lines: Despite having a similar PSII-core/LHCII ratio (Supplementary  
187 Figure S1), they also showed a few, yet much larger, grana with a diameter up to 3  $\mu\text{m}$  or even more (Figure  
188 4A-C), spanning the chloroplast sections, besides those alike observed wild type. We then proceeded to verify  
189 whether light exposure did affect thylakoid stacking. Dark-adapted leaves were illuminated with PSII light  
190 ( $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ,  $24^\circ\text{C}$ , 60 min) in order to promote PQ over-reduction despite the small PSII  
191 antenna size of both *Lhcb2-only* and *lowLHCII* genotypes, thus Lhcb2 phosphorylation (Figure 4D). The very  
192 in *Lhcb2-only* chloroplasts large grana essentially broke down upon illumination, and the size distribution  
193 extended towards narrower diameters. In both *Lhcb2-only* and *lowLHCII* lines, grana length and level of  
194 stacking were significantly reduced by state 2 induction (Figure 4E-F).

### 195 **Physiological consequences of thylakoid stacking for photosynthesis**

196 Chloroplasts of unicellular algae do not show well-defined grana stacks, while cyanobacteria exhibit single  
197 membranes, showing that oxygenic photosynthesis can proceed without the need of a stacked organization

198 of thylakoids (Mullineaux, 2005). Nevertheless, consequences of decreased level of stacking are clear from  
199 Figures 1 and 3: growth is dependent on the level of stacking, with the wild type showing both the maximal  
200 level of membrane stacking and the best growth. *NoM* and *koLHCII* exhibited intermediate level of both  
201 biomass yield and thylakoid stacking, while *koLhcb* has no grana and was severely impaired in growth. While  
202 these data clearly showed that growth rate and grana stacking were related, the functional reason(s) for the  
203 growth phenotype are still unclear. Grana stacking has been suggested to affect (1) light-harvesting, (2) the  
204 switch between CEF and LEF and/or (3) the repair of damaged PSII (Hepworth et al., 2021; Koochak et al.,  
205 2019; Mullineaux & Emllyn-Jones, 2005; Pribil et al., 2014). We proceeded to evaluate whether the PSII repair  
206 rate was affected by thylakoid stacking. To this aim, leaves were treated with excess light to induce  
207 photoinhibition, as detected by  $F_v/F_m$  ratio, which was reduced to about 30% of initial values in all genotypes.  
208 The recovery of  $F_v/F_m$  was then followed under low light conditions ( $15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ,  $24^\circ\text{C}$ ) favoring  
209 the PSII repair process (Mattoo et al., 1999). The results are shown in Figure 5A. Clearly, the wild type and  
210 *koLHCII* genotypes showed the same rate of PSII recovery. A similar dataset was published previously for the  
211 *NoM* genotype (Dall'Osto et al., 2020), showing no difference in the kinetic of recovery with respect to wild  
212 type plants. Instead, *koLhcb* healed its PSII activity to a much lower extent, suggesting that lateral  
213 heterogeneity between fully functional PSII in grana and PSII under repair in stroma-exposed margins might  
214 be necessary for a fully functional PSII repair cycle.

215 We then proceeded to verify whether the different level of thylakoid stacking did affect regulation of LEF vs.  
216 CEF amplitudes, a crucial mechanism to balance the chloroplast energy budget. To this aim, the kinetic of  
217  $P700^+/P700$  ratio was monitored in dark-adapted leaves upon far red illumination, when PSII has a far lower  
218 turnover rate than PSI. The slow P700 oxidation reflects a high CEF/LEF ratio, because the C3 cycle is inactive  
219 in dark-adapted leaves (Joliot & Joliot, 2005) and suggested the capacity to perform CEF in all genotypes. The  
220 procedure was repeated at different time intervals, up to 10 minutes upon exposure to actinic light, thus  
221 allowing for full rate LEF. When comparing the results from the wild type, *NoM*, *koLHCII* and *koLhcb*, it clearly  
222 appeared that the oxidation rate of P700 increased after sequential periods of priming with light, thus  
223 implying that LEF replaced CEF in all genotypes. However, *koLHCII* and *koLhcb* reached lower level of  
224  $P700^+/P700$  ratio than the wild type, and CEF-to-LEF transition occurred more slowly in *koLhcb* leaves, likely  
225 due to their reduced PSII antenna size (Figure 5B).

226 We then measured Chl fluorescence and the kinetic of electrochromic shift (ECS) on leaves, as a measure of  
227 electrons and protons transfer in the photosynthetic apparatus, respectively (Figure 5C). The quantum yield  
228 of PSII photochemistry can be used to measure the electron released from PSII (LEF) in leaves under a  
229 different light. ECS kinetics can be used to estimate fluxes of protons through the thylakoids, upon steady-  
230 state actinic illumination (Cruz et al., 2005):  $\text{ECS}_t$ , the total amplitude of the ECS signal upon light-dark  
231 transition, measures the light-driven protonmotive force (pmf) across the membrane, while the ECS decay  
232 lifetime ( $\text{gH}^+$ ) assesses the conductivity of the thylakoid ATPase to proton efflux. Under steady-state

233 photosynthetic electron transport, the pmf produced by LEF alone can be estimated by the LEF/gH<sup>+</sup> ratio, a  
234 parameter termed pmfLEF (Avenson et al., 2005). It turned out that the slope of the linear relationship of  
235 pmfLEF vs. ECS<sub>t</sub> was proportional to the stoichiometry of electron vs. proton transfer. Figure 5C shows that  
236 wild type and mutant plants produced approximately the same extent of pmf that can be accounted for by  
237 LEF only, thus suggesting a similar CEF amplitude in all genotypes.

238 Finally, the function of grana stacks, as for light-harvesting, consists in avoiding spill-over of excitons  
239 harvested by PSII-LHCII towards PSI-LHCI, which would unbalance the electron transport chain. This is  
240 obtained by partitioning PSI-LHCI and PSII-LHCII into two different domains of thylakoids. In order to verify  
241 the occurrence of spill-over, we have favored a 77K fluorescence spectroscopy approach. To this aim, we  
242 have compared fluorescence emission spectra of thylakoid membranes before and after treatment with very  
243 low concentrations of  $\alpha$ -DM (0,0055%), which disconnects PSI-LHCI complex from LHCII and/or PSII core  
244 complexes in stroma-exposed membranes without solubilization (Caffarri et al., 2014)(Bassi et al., 1985;  
245 Caffarri et al., 2014) (Figure 5A). Upon excitation of Chl *a* at 440 nm, the fluorescence-emission spectra of  
246 wild-type thylakoids showed three peaks at 686, 695-700 and 735 nm, respectively from Lhcb antenna  
247 proteins, PSII core and PSI-LHCI complexes. Emission by PSII can be described with two components, peaking  
248 at 684.5 nm for PSII antenna and 692 nm for core complex (Supplementary Figure S4). Upon  $\alpha$ -DM addition,  
249 the 695 nm shoulder component in 440 nm (Chl *a*) excited spectra was drastically reduced, with a  
250 concomitant increase of the 684 nm peak consistent with disruption of excitation energy transfer (EET) from  
251 LHCII to PSII and/or PSI. Analysis of spectra from *koLhcb* showed two peaks only, at 689 and 735 nm, from  
252 PSII core and PSI-LHCI, respectively. Since the 689 nm peak is weak and is enhanced by  $\alpha$ -DM, it can be  
253 concluded that PSII core was spilling-over excitation energy to PSI-LHCI and that this EET was severed by  $\alpha$ -  
254 DM. The analysis of spectra from *koLHCII* confirmed and extended the above conclusions: the untreated  
255 thylakoids showed a spectrum very similar to *koLhcb*. Since both components were enhanced (especially the  
256 685 nm peak from LHCs) upon  $\alpha$ -DM treatment, we explained the result as the release of quenching by PSI-  
257 LHCI on PSII-LHC complexes by  $\alpha$ -DM. The case of *NoM* is similar to that of the wild type: besides the broad  
258 peak at 735 nm, the major peak occurred at ~700 nm with a shoulder at 682 nm, a blue-shifted emission  
259 component previously ascribed to antennae badly connected to the core (Dall'Osto et al., 2014). Upon  $\alpha$ -DM,  
260 the 680 nm shoulder was strongly enhanced. This suggests that the  $\alpha$ -DM treatment further disconnected  
261 the EET from LHCII to PSII core although the effect appeared incomplete when compared with the wild type,  
262 as evidenced by the emission at 700 nm from Chl *a* binding core upon excitation at 440 nm.

263

## 264 **Discussion**

265 Grana stacks are the most conspicuous feature of plant chloroplasts, which attracted the attention of Mayer  
266 since 1883, when they were first reported (Gunning et al., 2006). The reasons for this interest is that grana  
267 are found in land plant, but are absent in algal chloroplast which, despite the obviously common function of

268 chloroplasts through the species, do not have this highly structured multiple membrane layers, but still have  
269 differentiation between stacked and unstacked membrane domains (Trissl & Wilhelm, 1993).

270 The deletion of either the inner layer (monomeric), or the outer layer (trimeric) antenna complexes, or both,  
271 had a strong impact on growth rate which was maximal with depletion of monomeric antennas, while the  
272 deletion of trimeric LHCII, although causing a moderate decrease in growth, did not affect biomass  
273 accumulation as much as it could be predicted by the difference in antenna size (Figure 1A,B). This is  
274 particularly evident when comparing the *NoM* with the wild type: since *NoM* strongly over-accumulates  
275 LHCII, its PSII antenna size, as determined by fluorescence induction with DCMU, is actually higher than the  
276 wild type and yet its growth is decreased by 50% (Figure 2B). We conclude that monomeric LHCs have an  
277 essential function in the efficiency of excitation energy transfer from outer antenna to RC of PSII, consistent  
278 with the high  $F_0$  values previously reported for this genotype (Dall'Osto et al., 2014). The higher-than-wild  
279 type PSII antenna size in *NoM* is at odd with its reduced growth, implying that factors other than ET to PSII  
280 RC are involved in growth retardation. The stronger loss of growth is observed in the absence of both the  
281 antenna types. Again, the loss of biomass is far higher than suggested by the apparent reduction antenna  
282 size, strengthening the above conclusion. Growth retardation does not appear to be due to enhanced  
283 photodamage, because increasing photon fluence by a factor of three (Figure 1B,D) did enhance the growth  
284 rate of all the genotypes except for the *koLhcb* plants, whose growth was independent on light intensity. It  
285 should be noted that the strongest gain in growth when comparing HL vs. LL was obtained in the *koLHCII*  
286 plants which retained monomeric LHCs (+80%), suggesting that these complexes are related to such growth  
287 promoting factors. The decrease in antenna size of the different phenotypes was associated to an higher  
288 PSII/PSI ratio (Figure 2A), a partial compensation for the reduced antenna size of PSII. Indeed, an enhanced  
289 PSII vs. PSI content and a low  $F_m/F_0$  ratio has been observed in the alga *Mantoniella squamata* that lacks  
290 grana stacks and thylakoid lateral heterogeneity because of a simplified antenna system composed of a single  
291 LHCII like protein (Rhiel & Mörschel, 1993), which is shared by PSI and PSII RCs, leading to spill-over of PSII  
292 excitation energy by PSI (Wilhelm et al., 1989).

293 Based on evidence from basic photosynthetic parameters and the lesson from comparative analysis of  
294 thylakoid lateral heterogeneity in *Mantoniella*, *Pleurochloris*, *Chlorella* and higher plants (Trissl & Wilhelm,  
295 1993), it could be hypothesized that by knocking out LHC genes we had both decreased the PSII antenna size  
296 and reduced the lateral heterogeneity between PSI and PSII, likely by causing de-stacking of thylakoid  
297 membranes.

298

299 **Thylakoid stacking.** Indeed, the analysis of thylakoid stacking confirmed that the growth rate correlated to  
300 the extent of thylakoid stacking (Figure 3K). The extent of membrane stacking was decreased by 30% in both

301 *NoM* and *koLHCII* plants, implying that components of both monomeric and trimeric LHC proteins were  
302 involved in stabilizing grana partitions. The deletion of both PSII antenna types has a tremendous effect, in  
303 that only agranal chloroplasts were detected. The relevance of monomeric vs trimeric LHCs in eliciting grana  
304 stacking can be assessed by comparing the *lowLHCII* genotype, containing mostly Lhcb1 with little Lhcb2 gene  
305 products, with the *koLHCII*, retaining monomeric complexes. These genotypes had the same overall total LHC  
306 antenna level. Since the *lowLHCII* plants had approximately 18% stacking as compared to the one retaining  
307 the three monomeric complexes (*koLHCII*), we conclude that the efficiency of monomeric antennas in causing  
308 stacking was 5-6 times higher with respect to trimeric LHCII. It had to be established whether the three inner  
309 antennae equally contributed to the stacking, or if this property was specific to one of them. To this aim, we  
310 took advantage of the early observation that the *ch1* mutant, in which a lack of Chl *b* de-stabilizes LHCs,  
311 retained a substantial level of stacking which closely matched the level found in *koLHCII* (Figure 3K) (Bassi et  
312 al., 1985). Loss of Chl *b* prevented accumulation of all LHC proteins, yet CP26 (Lhcb5) was retained (Havaux  
313 et al., 2004) because CP26 can binding Chl *a* at most of its Chl *b* binding sites (Croce et al., 2002). When the  
314 *ch1 koLhcb5* double mutant was analyzed for thylakoid stacking it showed, indeed, a sharp decrease (Figure  
315 3J), implying that Lhcb5 was a major determinant of grana formation. Last, but not less impressive, was the  
316 stacking phenotype of *Lhcb2-only* plants. Lhcb2 is present at low levels in thylakoids as compared to Lhcb1  
317 and yet it was shown to be essential for state 1 - state 2 transitions (Pietrzykowska et al., 2014). State 1 -  
318 state 2 transitions cause thylakoid de-stacking upon phosphorylation-dependent migration of LHCII trimers,  
319 containing both Lhcb1 and Lhcb2, from grana to stroma membranes (Bassi et al., 1989)(Hepworth et al.,  
320 2021). The huge grana stacks, far wider than in the wild type, evidenced in *Lhcb2-only* genotype (Fig. 4B)  
321 imply that Lhcb2 is prominent in mediating membrane appression into grana partitions. This was confirmed  
322 by the effect of inducing Lhcb2 phosphorylation with PSII light treatment (Figure 4D). Lhcb2 phosphorylation  
323 dramatically reduced the number of grana as well as their size (Figure 4B,C,E). We suggest that the grana  
324 remaining upon light treatment are promoted by un-phosphorylated LHCII. In a recent study, the formation  
325 of dimeric PSII supercomplexes connected by their stromal side was studied as a proxy for thylakoid stacking,  
326 by cross-linking and proteomic analysis. Consistent with our finding, both the monomeric and the trimeric  
327 antenna were suggested to mediate the interaction between PSII supercomplexes (Albanese et al., 2020).  
328 Nevertheless, the identity of the key gene products identified in that study (i.e. Lhcb1 and Lhcb4) does not  
329 match our *in vivo* analysis. Although these LHCs might well contribute to stacking, the genetic evidence we  
330 show here suggests that the major player in determining stacking and its regulation through phosphorylation  
331 are, indeed, the Lhcb5 and the Lhcb2 components of the LHCII trimeric antenna. This difference in attribution  
332 might be ascribed to the use of the purified dimeric PSII supercomplex fraction which, although reminiscent  
333 of face-to-face stacking, it might represent a PSII population resistant to detergent treatment yet not fully  
334 representative of the general PSII organization in grana partitions. Indeed, the same research group has  
335 recently reported on alternative organization forms of the face-to-face dimeric complexes (Grinzato et al.,

336 2020) and a recent high resolution study has identified two distinct conformations of the PSII C2S2 complex  
337 one of which is consistent with grana stacking being stabilized by interactions between CP26 and a LHCI  
338 subunit (Caspary et al., 2021). Alternatively, the differences with respect to our reports might well be ascribed  
339 to the *in vivo vs. in vitro* analysis: accessibility of the cross-linkers to individual LHC complexes might not  
340 correspond to the strength of interactions they elicit as thylakoid stacking determinants (A. Crepin & Caffarri,  
341 2018).

342 **Functional significance of the grana stacking.** In C3 plants and most green algae, the two PSs are well  
343 separated, making the possibility of exciton spilling from PSII antenna in a grana partition to PSI antenna  
344 pigments in the stroma-lamellae, rather small (Kirchhoff et al., 2007). This structural arrangement has the  
345 advantage of increasing the concentration of PSII, which is kinetically slower, by packing it densely in grana  
346 partitions and increasing the cooperativity between PSII RC beyond the obvious level of 2 defined by the  
347 dimeric organization of supercomplexes (Lavergne & Joliot, 1991; Lavergne & Trissl, 1995)(Lavergne & Trissl,  
348 1995). The high PSII/PSI stoichiometry ratio and high  $F_m/F_0$  ratio are in agreement with such good excitonic  
349 separation, which require state 1 - state 2 transitions in order to balance the turnover-rate when light is not  
350 equally absorbed by the two PSs (Miller & Lyon, 1985). Moreover, the requirement for the CBB cycle and  
351 other metabolic uses of ATP/NADPH from the light phase of photosynthesis, is variable. Indeed, changes in  
352 CET vs. LET rates have been proposed to depend on state transitions and their effect in modulating the ratio  
353 between grana and stroma membranes (Hepworth et al., 2021). Last but not least, PSII is found in its fully  
354 functional state in grana partitions, while its repair process upon photoinhibition occurs in stroma  
355 membranes, where the machinery for disassembly of PSII core complex is located.

356 Analysis of these physiological processes in dependence of the extent of grana stacking showed that the  
357 efficiency of PSII repair process, i.e. the kinetic of recovery from photoinhibition, was severely affected in  
358 *koLhcb* as compared to *koLHCII* (Figure 5A). This effect could be due to the lack of monomeric LHCs in the  
359 former genotype or to the lack of grana. We favor the second hypothesis because the *NoM* genotype,  
360 although lacking monomeric LHCs, like *koLhcb*, shows recovery kinetic from photoinhibition as in the wild  
361 type (Dall'Osto et al., 2020). Instead, we did not observe any major differences in CET vs. LET depending on  
362 the level of grana stacking, because all genotypes undergo CET to LET transition, although at a different rate  
363 (Figure 5B). We interpret the slower transition from CEF to LEF observed in *koLhcb* and, to a lower extent, in  
364 *koLHCII*, as caused by the strongly reduced antenna size of this genotype, limiting electron supply and  
365 reduction of P700<sup>+</sup>. A strong change in the CET/LET ratio should be reflected in the slope of the  
366 electrochromic shift signal vs. protonmotive force, which was not observed since all genotypes behave  
367 similarly in this respect (Figure 5C).

368 Lastly, we consider the possibility of excitation energy being spilled over from PSII to PSI upon thylakoid de-  
369 stacking. According to previous work with systems showing high spill-over, i.e. the liken *Parmelia sulcata*

370 (Slavov et al., 2013) and the primitive unicellular green alga *Mantoniella squamata* (Trissl & Wilhelm, 1993),  
371 a low  $F_m/F_0$  ratio associated to the high PSII/PSI stoichiometry is the best indication of ongoing spill-over, as  
372 assessed by fast fluorescence spectroscopy at 77K and DAS deconvolution (Slavov et al., 2013). We observed  
373 that *koLhcb* has a similarly low  $F_m/F_0$  and PSII/PSI ratio as in *Mantoniella* while genotypes retaining significant  
374 level of stacking were similar to the wild type, i.e had high  $F_m/F_0$  and PSII/PSI  $\sim 1$  (Supplementary Table S4).  
375 Furthermore, by treating thylakoids with sub-solubilizing concentration of  $\alpha$ -DM, which uncouples ET  
376 between pigment-proteins in thylakoids (Bassi et al., 1989), we observe a strong up-rise of PSI fluorescence  
377 emission bands in the genotypes (*NoM*, *koLHCII* and *koLhcb*) with a reduced level of stacking, while in the  
378 wild type the effect was very small, if any. The case of *koLhcb* is particularly clear, since these thylakoids only  
379 contain PSII core and PSI-LHCI complexes, without any PSII antenna (Figure 5A). It should be noted that the  
380 canonical method for assessing spill-over consists in showing an increase of P700 antenna size in DCMU-  
381 treated samples (Wilhelm et al., 1989). We have attempted this experiment with inconclusive results.  
382 Indeed, our mutants, particularly *koLHCB*, have a reduced cross section for photon absorption due to the very  
383 nature of the mutations applied: a PSII core complex binds 35 Chl a (Müh & Zouni, 2020) a figure that might  
384 be at the limit of detection for optically detected measurement since PSI-LHCI complex binds at least 145  
385 Chls plus several LHCII complexes (Schiphorst et al., 2021). In order to fully verify the hypothesis of spill-over  
386 occurring differentially in our grana endowed vs grana depleted genotypes, fast spectroscopy measurements  
387 need to be performed and will be the object of future studies. Yet the occurrence of this process in our grana-  
388 depleted genotypes is strongly consistent with data, namely the high  $F_m/F_0$  ratio of *Mantoniella squamata*  
389 (Rhiel & Mörschel, 1993) and other photosynthetic systems without well defined compartmentation between  
390 psi and PSII RC.

391 We conclude that thylakoid stacking is a characteristic of specific and lowly abundant Lhcb proteins, namely  
392 Lhcb5 (CP26) and, chiefly, Lhcb2. Indeed, while CP26 is conserved in algae, Lhcb2 cannot be traced back  
393 though the green lineage from land plants to green algae, which, in turn, do not show grana. As for the major  
394 physiological functions associated to the evolution of grana, it is a more efficient PSII repair process, possibly  
395 due to the compartmentalization of the fully functional PSII separately from the damaged complex under  
396 repair and the energy separation of PSI from PSII antenna. Indeed, the evolution of red forms upon land  
397 colonization has made the effect of spill-over far more deleterious for an equilibrated energy balance  
398 between PSs with respect to algae, whose PSI is depleted in red-forms and is, therefore, less efficient in  
399 draining excitation energy from connected antenna beds, according to Boltzmann's relation.

#### 400 **Aknowledgements**

401 We thank Pierre Joliot, Francis-André Wollman and Benjamin Bailleul for discussions and suggestions, the  
402 team of Electron Microscopy service at the University of Padua-department of Biology Vallisneri for excellent  
403 technical assistance. The work was supported by grant RIBA 2017 to R.B. and grant PRIN 2018 to L.D.

404

#### 405 **Author contributions**

406 R.B. and L.D. conceived the work and designed the experiments. R.B. performed E.M. analysis. Z.G. carried  
407 out the construction of mutants and performed their physiological characterization, together with L.D. and  
408 R.C. R.L.G. analyzed fluorescence kinetics and biochemically characterized mutant lines. J.S. developed and  
409 provided the CRISPR-Cas9 plasmids system. All authors contributed to writing the manuscript, discussed the  
410 results and commented on the manuscript.

411

#### 412 **Declaration of Interest**

413 The authors declare no competing interests.

414

#### 415 **Materials and Methods**

416

417 *Plant materials.* *Arabidopsis thaliana* mutants *ch1*, *ch1 koLhcb5*, *koLhcb3* and *NoM* were obtained as  
418 previously described (Dall'Osto et al., 2014; Damkjaer et al., 2009; Havaux et al., 2007). *NoM koLhcb3* was  
419 obtained by crossing single mutants and selecting progeny by immunoblotting. *koLHCII*, *Lhcb2-only*, *koLhcb*  
420 and *lowLHCII* lines were obtained by GE as reported in (Ordon et al., 2017, 2020). Plants were transformed  
421 (Zhang et al., 2006) with *Agrobacterium tumefaciens* (strain GV3101). Seedlings were tested for resistance to  
422 applications of the antibiotic hygromycin (25 mg l<sup>-1</sup>). For each genotype, independent transformants (T1  
423 generation) were self-fertilized, and absences of proteins were confirmed in the T3 generation by  
424 immunotitration.

425 *Growth conditions.* Wild type and mutant genotypes were grown in soil, in a phytotron for 6 weeks at either  
426 150 or 350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 23°C, 70% relative humidity, 8/16 h of day/night. All biochemical and  
427 physiological analyses were performed on plants prior to the onset of flowering. The growth of plants was  
428 determined by measuring the rosette fresh weight at the end of the growth cycle.

429 *Membrane isolation.* Stacked thylakoid membranes were isolated as previously described (Casazza et al.,  
430 2001).

431 *Pigment analysis.* Pigments were extracted from leaf discs with 85% acetone buffered with Na<sub>2</sub>CO<sub>3</sub>, then  
432 separated and quantified by HPLC (Jasco Extrema LC-4000) as in (Gilmore & Yamamoto, 1991).

433 *Electrophoresis and immunoblotting.* SDS-PAGE analysis of thylakoid proteins was performed using the Tris-  
434 Tricine buffer system (Schägger & von Jagow, 1987). For immunotitration (Towbin et al., 1979), proteins were  
435 detected with alkaline phosphatase-conjugated antibody (Sigma-Aldrich A3687). Primary antibodies used  
436 were:  $\alpha$ -PsbB/CP47 (AS04 038),  $\alpha$ -Lhcb1 (AS01 004),  $\alpha$ -Lhcb2 (AS01 003),  $\alpha$ -P-Lhcb1 (AS13 2704),  $\alpha$ -P-Lhcb2  
437 (AS13 2705) from Agrisera. Signal amplitude was quantified using the GelPro 3.2 software (Bio-Rad). Non-  
438 denaturing Deriphath-PAGE was performed as in (Guardini et al., 2020). SDS-PAGE analysis on complexes

439 eluted from non-denaturing gel was performed using the Laemmli system as reported in (Aurélie Crepin et  
440 al., 2020).

441 *Spectroscopy.* Absorption spectra were recorded at RT using a Jasco V-550 spectrophotometer. Leaf pigment  
442 content was calculated from spectra of acetonic extracts (Croce et al., 2002). Absorption measurements on  
443 complexes eluted from non-denaturing gel were performed in 5% glycerol, 10 mM HEPES pH 7.5,  $\alpha$ -DM  
444 0.004%. 77K fluorescence emission spectra ( $\lambda_{exc}$  440 nm,  $\sim 0.2 \mu\text{g Chl mL}^{-1}$  in 50% glycerol, 10 mM HEPES, pH  
445 7.5) were recorded using a Jobin-Yvon Fluoromax-3 spectrofluorometer (Dall'Osto et al., 2017).

446 *Analysis of Chl fluorescence.* Photosynthetic parameters (Baker, 2008) were measured on leaves at RT with a  
447 PAM 101 fluorimeter (Heinz-Walz). Changes in the redox state of P700 and CEF to LEF transition were  
448 measured as in (Dal Corso et al., 2008) with a DUAL-PAM-100 equipment (Walz, GmbH). Variable  
449 fluorescence was measured in a home-built Chl fluorimeter, and induced by green light (Rappaport et al.,  
450 2007) ( $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in dark adapted leaves, infiltrated with DCMU 50  $\mu\text{M}$ . Steady state, light-  
451 induced pmf was estimated from changes in absorbance associated with the ECS at 520 nm (Cruz et al., 2005;  
452 Livingston et al., 2010), using a LED spectrophotometer (JTS10; Biologic Science Instruments).

453 *Electron Microscopy and image analysis.* Transmission electron microscopy on leaf fragments was conducted  
454 using a FEI Tecnai T12 electron microscope operating at 100 kV accelerating voltage. Leaf fragments were  
455 fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer pH 6.9. Analyses on EM images were conducted with  
456 ImageJ software (Schneider et al., 2012).

457 *LHCII phosphorylation induction.* Leaves were dark adapted for 60 minutes prior to exposure to 200  $\mu\text{mol}$   
458  $\text{photons m}^{-2} \text{s}^{-1}$  PSII light (orange) for 60 minutes at 22°C.

459 *Statistics.* Statistical analyses were performed in SigmaPlot using One-way analysis of variance (ANOVA),  
460 means were separated with Tukey's post hoc test at a significant level of  $P < 0.05$  (see the figure legends for  
461 details). Error bars represent the standard deviation.

462 *Data availability.* Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or  
463 GenBank/EMBL databases under accession numbers At1g29920 (*Lhcb1.1*), At1g29910 (*Lhcb1.2*), At1g29930  
464 (*Lhcb1.3*), At2g34430 (*Lhcb1.4*), At2g34420 (*Lhcb1.5*), At2g05100 (*Lhcb2.1*), At2g05070 (*Lhcb2.2*), At3g27690  
465 (*Lhcb2.3*), At5g54270 (*Lhcb3*), At5g01530 (*Lhcb4.1*), At3g08940 (*Lhcb4.2*), At4g10340 (*Lhcb5*), At1g44446  
466 (*cao*). The KO lines used in the work were obtained from the NASC under the stock numbers N376476  
467 (*koLhcb4.1*), N877954 (*koLhcb4.2*), N514869 (*koLhcb5*), N520342 (*koLhcb3*), N524295 (*ch1*).

468

469

470

471

472

473

474 **References**

- 475 Albanese, P., Tamara, S., Saracco, G., Scheltema, R. A., & Pagliano, C. (2020). How paired PSII–LHCII  
476 supercomplexes mediate the stacking of plant thylakoid membranes unveiled by structural mass-  
477 spectrometry. *Nature Communications*, *11*(1). <https://doi.org/10.1038/s41467-020-15184-1>
- 478 Allred, D. R., & Staehelin, L. A. (1985). Lateral distribution of the cytochrome b6/f and coupling factor ATP-  
479 synthetase complexes of chloroplast thylakoid membranes. *Plant Physiol.*, *78*, 199–202.
- 480 Anderson, J. M., & Melis, A. (1983). Localization of different photosystems in separate regions of  
481 chloroplast membranes. *Proc.Natl.Acad.Sci.USA*, *80*, 745–749.
- 482 Argyroudi-Akoyunoglou, J. H., Feleki, Z., & Akoyunoglou, G. (1971). Formation of two chlorophyll-protein  
483 complexes during greening of etiolated bean leaves. *Biochemical and Biophysical Research  
484 Communications*, *45*(3), 606–614. [https://doi.org/10.1016/0006-291X\(71\)90460-8](https://doi.org/10.1016/0006-291X(71)90460-8)
- 485 Armond, P. A., & Arntzen, C. J. (1977). Localization and Characterization of Photosystem II in Grana and  
486 Stroma Lamellae. *Plant Physiology*, *59*(3), 398–404. <https://doi.org/10.1104/pp.59.3.398>
- 487 Armond, P. A., Arntzen, C. J., Briantais, J. M., & Verotte, C. (1976). Differentiation of chloroplast lamellae.  
488 Light harvesting efficiency and grana development. *Archives of Biochemistry and Biophysics*, *175*(1),  
489 54–63. [https://doi.org/10.1016/0003-9861\(76\)90484-7](https://doi.org/10.1016/0003-9861(76)90484-7)
- 490 Armond, P. A., Staehelin, L. A., & Arntzen, C. J. (1977). Spatial relationship of photosystem I, photosystem II,  
491 and the light-harvesting complex in chloroplast membranes. *J.Cell Biol.*, *73*, 400–418.
- 492 Avenson, T. J., Cruz, J. A., Kanazawa, A., & Kramer, D. M. (2005). Regulating the proton budget of higher  
493 plant photosynthesis. *Proceedings of the National Academy of Sciences of the United States of  
494 America*, *102*(27), 9709–9713. <https://doi.org/10.1073/pnas.0503952102>
- 495 Baker, N. R. (2008). Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo. *Annual Review of Plant  
496 Biology*, *59*(1), 89–113. <https://doi.org/10.1146/annurev.arplant.59.032607.092759>
- 497 Bassi, R., Ghiretti Magaldi, A., Tognon, G., Giacometti, G. M., & Miller, K. R. (1989). Two-dimensional  
498 crystals of the photosystem II reaction center complex from higher plants. *European Journal of Cell  
499 Biology*, *50*(1), 84–93. <https://europepmc.org/article/med/2693092>
- 500 Bassi, R., Marquardt, J., & Lavergne, J. (1995). Biochemical and functional properties of photosystem II in  
501 agranal membranes from maize mesophyll and bundle sheath chloroplasts. *Eur.J.Biochem.*, *233*, 709–  
502 719.
- 503 Bassi, R., & Simpson, D. J. (1986). Differential expression of LHCII genes in mesophyll and bundle sheath  
504 cells of maize. *Carlsberg Research Communications*, *51*(6), 363–370.  
505 <https://doi.org/10.1007/BF02907312>
- 506 Bassi, R., Hinz, U., & Barbato, R. (1985). The role of the light harvesting complex and photosystem II in  
507 thylakoid stacking in the chlorina-f2 barley mutant. *Carlsberg Res.Commun.*, *50*\_\_, 347–367.
- 508 Belgio, E., Kapitonova, E., Chmeliov, J., Duffy, C. D. P., Ungerer, P., Valkunas, L., & Ruban, A. V. (2014).

509 Economic photoprotection in photosystem II that retains a complete light-harvesting system with slow  
510 energy traps. *Nature Communications*, 5, 1–8. <https://doi.org/10.1038/ncomms5433>

511 Caffarri, S., Tibiletti, T., Jennings, R., & Santabarbara, S. (2014). A Comparison Between Plant Photosystem I  
512 and Photosystem II Architecture and Functioning. *Current Protein & Peptide Science*, 15(4), 296–331.  
513 <https://doi.org/10.2174/1389203715666140327102218>

514 Campoli, C., Caffarri, S., Svensson, J. T., Bassi, R., Stanca, A. M., Cattivelli, L., & Crosatti, C. (2009). Parallel  
515 pigment and transcriptomic analysis of four barley Albina and Xantha mutants reveals the complex  
516 network of the chloroplast-dependent metabolism. *Plant Molecular Biology*, 71(1–2), 173–191.

517 Casazza, A. P., Tarantino, D., & Soave, C. (2001). Preparation and functional characterization of thylakoids  
518 from *Arabidopsis thaliana*. *Photosynth.Res.*, 68(0166-8595 (Print)), 175–180.  
519 <https://doi.org/10.1023/A:1011818021875>

520 Caspy, I., Fadeeva, M., Mazor, Y., & Nelson, N. (2021). *Structure of Dunaliella Photosystem II reveals*  
521 *conformational flexibility of stacked and unstacked supercomplexes*.  
522 <https://doi.org/10.1101/2021.11.29.470333>

523 Crepin, A., & Caffarri, S. (2018). Functions and evolution of Lhcb isoforms composing LHCII, the major light  
524 harvesting complex of Photosystem II of green eukaryotic organisms. *Current Protein & Peptide*  
525 *Science*, 19. <https://doi.org/10.2174/1389203719666180222101534>

526 Crepin, Aurélie, Kučerová, Z., Kosta, A., Durand, E., & Caffarri, S. (2020). Isolation and characterization of a  
527 large photosystem I–light-harvesting complex II supercomplex with an additional Lhca1–a4 dimer in  
528 *Arabidopsis*. *Plant Journal*, 102(2), 398–409. <https://doi.org/10.1111/tpj.14634>

529 Croce, R., Canino, G., Ros, F., & Bassi, R. (2002). Chromophore organization in the higher-plant photosystem  
530 II antenna protein CP26. *Biochemistry*, 41(23), 7334–7343. <https://doi.org/10.1021/bi0257437>

531 Cruz, J. A., Avenson, T. J., Kanazawa, A., Takizawa, K., Edwards, G. E., & Kramer, D. M. (2005). Plasticity in  
532 light reactions of photosynthesis for energy production and photoprotection. *Journal of Experimental*  
533 *Botany*, 56(411), 395–406. <https://doi.org/10.1093/jxb/eri022>

534 Dal Corso, G., Pesaresi, P., Masiero, S., Aseeva, E., Schünemann, D., Finazzi, G., Joliot, P., Barbato, R., &  
535 Leister, D. (2008). A Complex Containing PGRL1 and PGR5 Is Involved in the Switch between Linear  
536 and Cyclic Electron Flow in *Arabidopsis*. *Cell*, 132(2), 273–285.  
537 <https://doi.org/10.1016/j.cell.2007.12.028>

538 Dall’Osto, L., Cazzaniga, S., Bressan, M., Paleček, D., Židek, K., Niyogi, K. K., Fleming, G. R., Zigmantas, D., &  
539 Bassi, R. (2017). Two mechanisms for dissipation of excess light in monomeric and trimeric light-  
540 harvesting complexes. *Nature Plants*, 3(April), 17033. <https://doi.org/10.1038/nplants.2017.33>

541 Dall’Osto, L., Cazzaniga, S., Zappone, D., & Bassi, R. (2020). Monomeric light harvesting complexes enhance  
542 excitation energy transfer from LHCII to PSII and control their lateral spacing in thylakoids. *Biochimica*  
543 *et Biophysica Acta - Bioenergetics*, 1861(4), 0–1. <https://doi.org/10.1016/j.bbabi.2019.06.007>

544 Dall'Osto, L., Ünlü, C., Cazzaniga, S., & Van Amerongen, H. (2014). Disturbed excitation energy transfer in  
545 *Arabidopsis thaliana* mutants lacking minor antenna complexes of photosystem II. *BBA -*  
546 *Bioenergetics*, *1837*(12), 1981–1988. <https://doi.org/10.1016/j.bbabi.2014.09.011>

547 Damkjaer, J., Kereiche, S., Johnson, M. P., Kovacs, L., a.z Kiss, Boekema, E. J., Ruban, A. V, Horton, P., &  
548 Jansson, S. (2009). The Photosystem II light-harvesting protein Lhcb3 affects the macrostructure of  
549 Photosystem II and the rate of state transitions in *Arabidopsis*. *Plant Cell*, *21*, 3245–3256.

550 Gilmore, A. M., & Yamamoto, H. Y. (1991). Zeaxanthin Formation and Energy-Dependent Fluorescence  
551 Quenching in Pea Chloroplasts under Artificially Mediated Linear and Cyclic Electron Transport1. *Plant*  
552 *Physiology*, *96*(2), 635–643. <https://doi.org/10.1104/pp.96.2.635>

553 Granick, S., & Porter, K. R. (1947). THE STRUCTURE OF THE SPINACH CHLOROPLAST AS INTERPRETED WITH  
554 THE ELECTRON MICROSCOPE. *American Journal of Botany*, *34*(10), 545–550.  
555 <https://doi.org/10.1002/J.1537-2197.1947.TB13028.X>

556 Grinzato, A., Albanese, P., Marotta, R., Swuenc, P., Saracco, G., Bolognesi, M., Zanotti, G., & Pagliano, C.  
557 (2020). High-Light versus Low-Light: Effects on Paired Photosystem II Supercomplex Structural  
558 Rearrangement in Pea Plants. *International Journal of Molecular Sciences*, *21*(22), 8643.  
559 <https://doi.org/10.3390/ijms21228643>

560 Guardini, Z., Bressan, M., Caferrri, R., Bassi, R., & Dall'Osto, L. (2020). Identification of a pigment cluster  
561 catalysing fast photoprotective quenching response in CP29. *Nature Plants*, *6*(3), 303–313.  
562 <https://doi.org/10.1038/s41477-020-0612-8>

563 Gunning, B., Koenig, F., & Govindjee. (2006). A Dedication to Pioneers of Research on Chloroplast Structure.  
564 In R. R. Wise & J. K. Hooper (Eds.), *The structure and function of plastids* (1st ed., pp. xxiii–xxxi).  
565 Springer.

566 Havaux, M., Dall'Osto, L., & Bassi, R. (2007). Zeaxanthin has enhanced antioxidant capacity with respect to  
567 all other xanthophylls in *Arabidopsis* leaves and functions independent of binding to PSII antennae.  
568 *Plant Physiol*, *145*(0032-0889 (Print)), 1506–1520.

569 Havaux, M., Dall'Osto, L., Cui n , S., Giuliano, G., & Bassi, R. (2004). The Effect of Zeaxanthin As the only  
570 Xanthophyll on the Structure and Function of the Photosynthetic Apparatus in *Arabidopsis thaliana*.  
571 *Journal of Biological Chemistry*, *279*(14), 13878–13888. <https://doi.org/10.1074/jbc.M311154200>

572 Heinz, S., Rast, A., Shao, L., Gutu, A., G gel, I. L., Heyno, E., Labs, M., Rengstl, B., Viola, S., Nowaczyk, M. M.,  
573 Leister, D., & Nickelsen, J. (2016). Thylakoid membrane architecture in *Synechocystis* depends on  
574 CurT, a homolog of the granal CURVATURE THYLAKOID1 proteins. *Plant Cell*, *28*(9), 2238–2260.  
575 <https://doi.org/10.1105/tpc.16.00491>

576 Heitz, E. (1936). Untersuchungen  ber den Bau der Plastiden. *Planta* *1936* *26*:1, *26*(1), 134–163.  
577 <https://doi.org/10.1007/BF01913844>

578 Hepworth, C., Wood, W. H. J., Emrich-Mills, T. Z., Proctor, M. S., Casson, S., & Johnson, M. P. (2021).

579 Dynamic thylakoid stacking and state transitions work synergistically to avoid acceptor-side limitation  
580 of photosystem I. *Nature Plants*, 7(1), 87–98. <https://doi.org/10.1038/s41477-020-00828-3>

581 Iwai, M., Roth, M. S., & Niyogi, K. K. (2018). Subdiffraction-resolution live-cell imaging for visualizing  
582 thylakoid membranes. *Plant Journal*, 96(1), 233–243. <https://doi.org/10.1111/tpj.14021>

583 Joliot, P., & Joliot, A. (2005). Quantification of cyclic and linear flows in plants. *Proc.Natl.Acad.Sci.U.S.A.*,  
584 102(0027-8424 (Print)), 4913–4918.

585 Kim, E. H., Li, X. P., Razeghifard, R., Anderson, J. M., Niyogi, K. K., Pogson, B. J., & Chow, W. S. (2009). The  
586 multiple roles of light-harvesting chlorophyll a/b-protein complexes define structure and optimize  
587 function of Arabidopsis chloroplasts: A study using two chlorophyll b-less mutants. *Biochimica et*  
588 *Biophysica Acta - Bioenergetics*, 1787(8), 973–984. <https://doi.org/10.1016/j.bbabi.2009.04.009>

589 Kirchhoff, H. (2019). Chloroplast ultrastructure in plants. *New Phytologist*, 223(2), 565–574.  
590 <https://doi.org/10.1111/NPH.15730>

591 Kirchhoff, H., Haase, W., Haferkamp, S., Schott, T., Borinski, M., Kubitscheck, U., & Rögner, M. (2007).  
592 Structural and functional self-organization of Photosystem II in grana thylakoids. *Biochimica et*  
593 *Biophysica Acta (BBA) - Bioenergetics*, 1767(9), 1180–1188.  
594 <https://doi.org/10.1016/J.BBABI.2007.05.009>

595 Kirchhoff, H., Hall, C., Wood, M., Herbstová, M., Tsabari, O., Nevo, R., Charuvi, D., Shimoni, E., & Reich, Z.  
596 (2011). Dynamic control of protein diffusion within the granal thylakoid lumen. *Proceedings of the*  
597 *National Academy of Sciences of the United States of America*, 108(50), 20248–20253.  
598 <https://doi.org/10.1073/pnas.1104141109>

599 Koochak, H., Puthiyaveetil, S., Mullendore, D. L., Li, M., & Kirchhoff, H. (2019). The structural and functional  
600 domains of plant thylakoid membranes. *Plant Journal*, 97(3), 412–429.  
601 <https://doi.org/10.1111/tpj.14127>

602 Kramer, D. M., & Evans, J. R. (2011). The importance of energy balance in improving photosynthetic  
603 productivity. *Plant Physiology*, 155(1), 70–78. <https://doi.org/10.1104/pp.110.166652>

604 Kyle, D. J., Staehelin, L. A., & Arntzen, C. J. (1983). Lateral mobility of the light-harvesting complex in  
605 chloroplast membranes controls excitation energy distribution in higher plants.  
606 *Arch.Biochem.Biophys.*, 222, 527–541.

607 Lavergne, J., & Joliot, P. (1991). Restricted Diffusion in Photosynthetic Membranes. *Trends Biochem.Sci.*, 16,  
608 129–134.

609 Lavergne, J., & Trissl, H. W. (1995). Theory of fluorescence induction in photosystem II: derivation of  
610 analytical expressions in a model including exciton-radical-pair equilibrium and restricted energy  
611 transfer between photosynthetic units. *Biophysical Journal*, 68(6), 2474–2492.  
612 [https://doi.org/10.1016/S0006-3495\(95\)80429-7](https://doi.org/10.1016/S0006-3495(95)80429-7)

613 Livingston, A. K., Cruz, J. A., Kohzuma, K., Dhingra, A., & Kramer, D. M. (2010). An arabidopsis mutant with

614 high cyclic electron flow around photosystem i (hcef) involving the nadphdehydrogenase complex.  
615 *Plant Cell*, 22(1), 221–233. <https://doi.org/10.1105/tpc.109.071084>

616 Malzahn, A., Lowder, L., & Qi, Y. (2017). Plant genome editing with TALEN and CRISPR. *Cell and Bioscience*,  
617 7(1), 1–18. <https://doi.org/10.1186/s13578-017-0148-4>

618 Margulies, M. M. (1966). Effect of Chloramphenicol on Formation of Chloroplast Structure and Protein  
619 During Greening of Etiolated Leaves of *Phaseolus vulgaris*. *Plant Physiology*, 41(6), 992–1003.  
620 <https://doi.org/10.1104/pp.41.6.992>

621 Mattoo, A. K., Giardi, M.-T., Raskind, A., & Edelman, M. (1999). Dynamic metabolism of photosystem II  
622 reaction center proteins and pigments. *Physiologia Plantarum*, 107(4), 454–461.  
623 <https://doi.org/10.1034/J.1399-3054.1999.100412.X>

624 Mattoo, A., K., Marder, J. B., & Edelman, M. (1989). Dynamics of the photosystem II reaction center. *Cell*,  
625 56, 241–246.

626 Miller, K. R., & Lyon, M. K. (1985). Do we really know why chloroplast membranes stack? *Trends in*  
627 *Biochemical Sciences*, 10(6), 219–222. [https://doi.org/10.1016/0968-0004\(85\)90132-X](https://doi.org/10.1016/0968-0004(85)90132-X)

628 Müh, F., & Zouni, A. (2020). Structural basis of light-harvesting in the photosystem II core complex. *Protein*  
629 *Science*, 29(5), 1090–1119. <https://doi.org/10.1002/pro.3841>

630 Mullineaux, C. W. (2005). Function and evolution of grana. *Trends in Plant Science*, 10(11), 521–525.  
631 <https://doi.org/10.1016/j.tplants.2005.09.001>

632 Mullineaux, C. W., & Emlyn-Jones, D. (2005). State transitions: An example of acclimation to low-light  
633 stress. *Journal of Experimental Botany*, 56(411), 389–393. <https://doi.org/10.1093/jxb/eri064>

634 Murata, N. (1969). Control of excitation transfer in photosynthesis. I. Light- induced change of chlorophyll a  
635 fluorescence in *Porphyridium cruentum*. *Biochim.Biophys.Acta*, 172, 242–251.

636 Olive, J., Vallon, O., Wollman, F. A., Recouvreur, M., & Bennoun, P. (1986). Studies on the Cytochrome B6/F  
637 Complex .2. Localization of the Complex in the Thylakoid Membranes from Spinach and  
638 *Chlamydomonas-Reinhardtii* by Immunocytochemistry and Freeze-Fracture Analysis of B6/F Mutants.  
639 *Biochimica et Biophysica Acta*, 851(2), 239–248.

640 Ordon, J., Bressan, M., Kretschmer, C., Dall’Osto, L., Marillonnet, S., Bassi, R., & Stuttmann, J. (2020).  
641 Optimized Cas9 expression systems for highly efficient Arabidopsis genome editing facilitate isolation  
642 of complex alleles in a single generation. *Functional and Integrative Genomics*, 20(1), 151–162.  
643 <https://doi.org/10.1007/s10142-019-00665-4>

644 Ordon, J., Gantner, J., Kemna, J., Schwalgun, L., Reschke, M., Streubel, J., Boch, J., & Stuttmann, J. (2017).  
645 Generation of chromosomal deletions in dicotyledonous plants employing a user-friendly genome  
646 editing toolkit. *Plant Journal*, 89(1), 155–168. <https://doi.org/10.1111/tpj.13319>

647 Paolillo, D. J. J., & Reighard, J. A. (2011). ON THE RELATIONSHIP BETWEEN MATURE STRUCTURE AND  
648 ONTOGENY IN THE GRANA OF CHLOROPLASTS. <https://doi.org/10.1139/B67-083>, 45(6), 773–782.

649 <https://doi.org/10.1139/B67-083>

650 Pietrzykowska, M., Suorsa, M., Semchonok, D. A., Tikkanen, M., Boekema, E. J., Aro, E. M., & Jansson, S.  
651 (2014). The light-harvesting chlorophyll a/b binding proteins Lhcb1 and Lhcb2 play complementary  
652 roles during state transitions in Arabidopsis. *Plant Cell*, 26(9), 3646–3660.  
653 <https://doi.org/10.1105/tpc.114.127373>

654 Pribil, M., Labs, M., & Leister, D. (2014). Structure and dynamics of thylakoids in land plants. In *Journal of*  
655 *Experimental Botany* (Vol. 65, Issue 8, pp. 1955–1972). J Exp Bot. <https://doi.org/10.1093/jxb/eru090>

656 Rappaport, F., Béal, D., Joliot, A., & Joliot, P. (2007). *On the advantages of using green light to study*  
657 *fluorescence yield changes in leaves*. 1767, 56–65. <https://doi.org/10.1016/j.bbabi.2006.10.002>

658 Rhiel, E., & Mörschel, E. (1993). The atypical chlorophyll a/b/c light-harvesting complex of *Mantoniella*  
659 *squamata*: molecular cloning and sequence analysis. *Molecular and General Genetics MGG 1993*  
660 240:3, 240(3), 403–413. <https://doi.org/10.1007/BF00280392>

661 Schägger, H., & von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for  
662 the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, 166(2), 368–379.  
663 [https://doi.org/10.1016/0003-2697\(87\)90587-2](https://doi.org/10.1016/0003-2697(87)90587-2)

664 Schiphorst, C., Achterberg, L., Go, R., Koehorst, R., Bassi, R., Amerongen, H. Van, Osto, L. D., Wientjes, E., &  
665 Biotechnologie, D. (2021). *The role of light-harvesting complex I in excitation energy transfer from LHClI*  
666 *to photosystem I in Arabidopsis*. 1–12.

667 Schiphorst, C., & Bassi, R. (2020). *Chlorophyll-Xanthophyll Antenna Complexes: In Between Light Harvesting*  
668 *and Energy Dissipation*. 27–55. [https://doi.org/10.1007/978-3-030-33397-3\\_3](https://doi.org/10.1007/978-3-030-33397-3_3)

669 Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). *NIH Image to ImageJ: 25 years of image analysis*.  
670 <https://doi.org/10.1038/nmeth.2089>

671 Simpson, D. J. (1982). Freeze-fracture studies on barley plastid membranes V. viridis-n 34, a photosystem I  
672 mutant. *Carlsberg Research Communications 1982 47:4*, 47(4), 215–225.  
673 <https://doi.org/10.1007/BF02907873>

674 Simpson, D. J., & Robinson, S. P. (1984). Freeze-Fracture Ultrastructure of Thylakoid Membranes in  
675 Chloroplasts from Manganese-Deficient Plants. *Plant Physiology*, 74(3), 735–741.  
676 <https://doi.org/10.1104/PP.74.3.735>

677 Simpson, D. J., Vallon, O., & von Wettstein, D. (1989). Freeze-fracture studies on barley plastid membranes:  
678 VIII. In viridis-115, a mutant completely lacking Photosystem II, oxygen evolution enhancer 1 (OEE1)  
679 and the  $\alpha$ -subunit of cytochrome b-559 accumulate in appressed thylakoids. *Biochimica et Biophysica*  
680 *Acta (BBA) - Bioenergetics*, 975(1), 164–174. [https://doi.org/10.1016/S0005-2728\(89\)80215-4](https://doi.org/10.1016/S0005-2728(89)80215-4)

681 Slavov, C., Reus, M., & Holzwarth, A. R. (2013). Two Different Mechanisms Cooperate In The Desiccation-  
682 Induced Excited State Quenching In *Parmelia* Lichen. *Journal of Physical Chemistry B*, 117(38), 11326–  
683 11336. <https://doi.org/10.1021/JP402881F>

684 Standfuss, R., van Scheltinga, A. C. T., Lamborghini, M., & Kuhlbrandt, W. (2005). Mechanisms of  
685 photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5Å resolution.  
686 *Embo Journal*, 24(5), 919–928.

687 Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels  
688 to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of*  
689 *Sciences*, 76(9), 4350–4354. <https://doi.org/10.1073/pnas.76.9.4350>

690 Trissl, H. W., & Wilhelm, C. (1993). Why do thylakoid membranes from higher plants form grana stacks?  
691 *Trends in Biochemical Sciences*, 18(11), 415–419. [https://doi.org/10.1016/0968-0004\(93\)90136-B](https://doi.org/10.1016/0968-0004(93)90136-B)

692 Vallon, O., Bulte, L., Dainese, P., Olive, J., Bassi, R., & Wollman, F. A. (1991). Lateral redistribution of  
693 cytochrome b6/f complexes along thylakoid membranes upon state transitions.  
694 *Proc.Natl.Acad.Sci.U.S.A*, 88(0027-8424 SB-IM), 8262–8266.

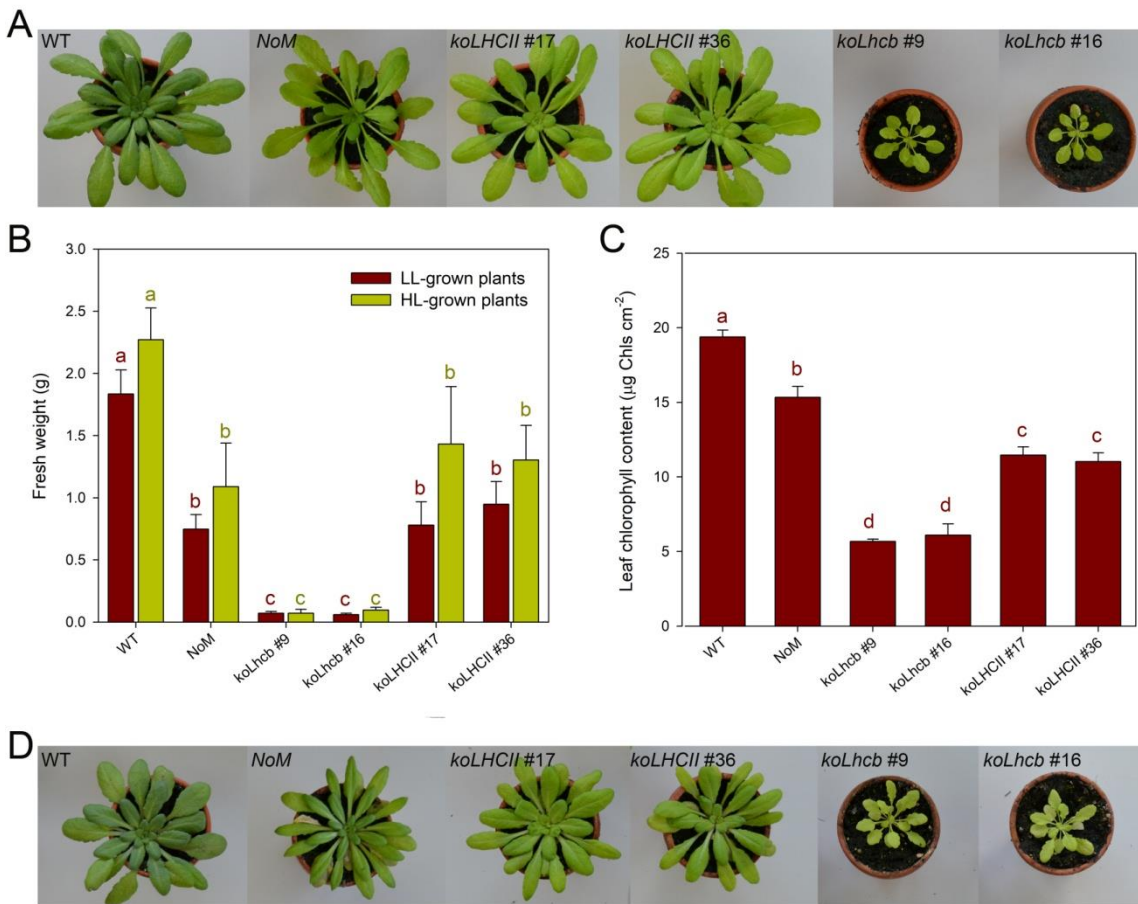
695 Wietrzynski, W., Schaffer, M., Tegunov, D., Albert, S., Kanazawa, A., Plitzko, J. M., Baumeister, W., & Engel,  
696 B. D. (2020). Charting the native architecture of Chlamydomonas thylakoid membranes with single-  
697 molecule precision. *ELife*, 9, 1–18. <https://doi.org/10.7554/eLife.53740>

698 Wilhelm, C., Krämer, P., & Lenartz-Weiler, I. (1989). The energy distribution between the photosystems and  
699 light-induced changes in the stoichiometry of system I and II reaction centers in the chlorophyll b-  
700 containing alga *Mantoniella squamata* (Prasinophyceae). *Photosynthesis Research 1989 20:3*, 20(3),  
701 221–233. <https://doi.org/10.1007/BF00034066>

702 Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., Chua, N.-H., Niu, W.-W., Chua, N.-H., Niu, Q.-W., & Chua, N.-  
703 H. H. N.-H. (2006). Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral  
704 dip method. *Nature Protocols*, 1(2), 641–646. <https://doi.org/10.1038/nprot.2006.97>

705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718

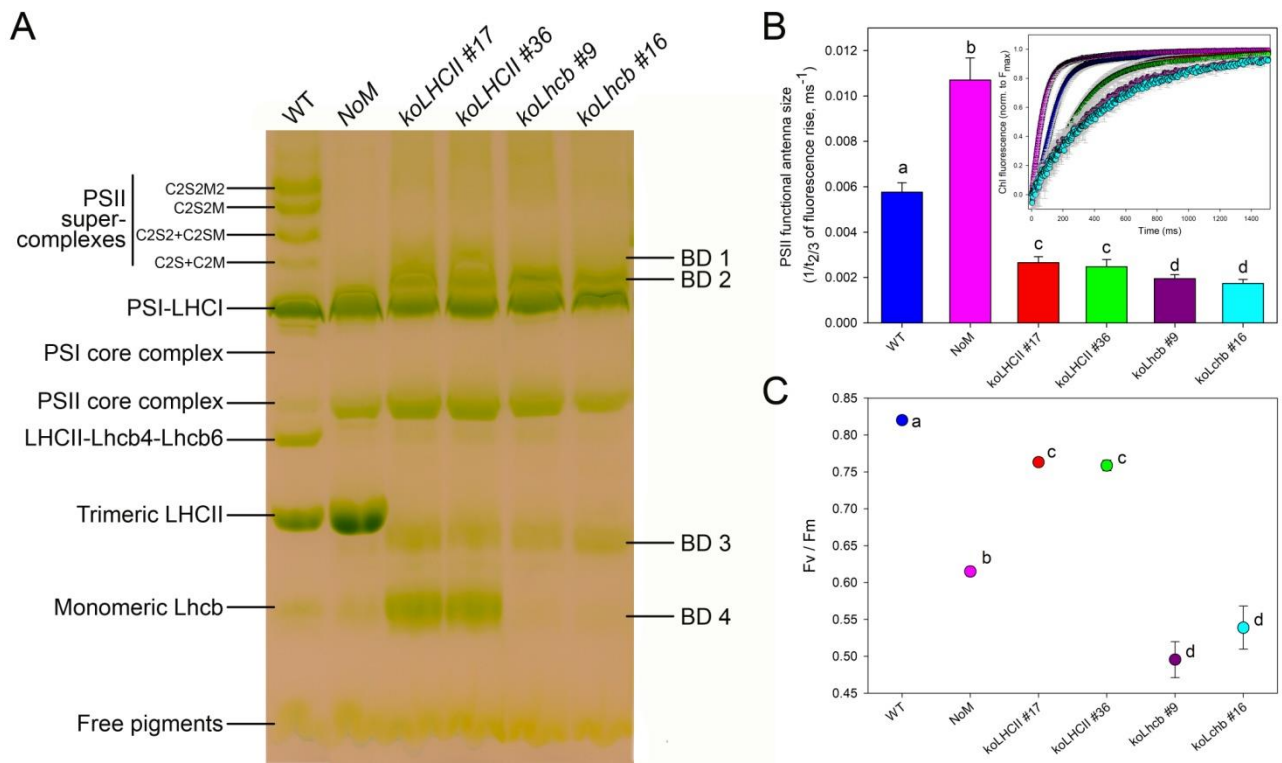
719 **Figures**



720

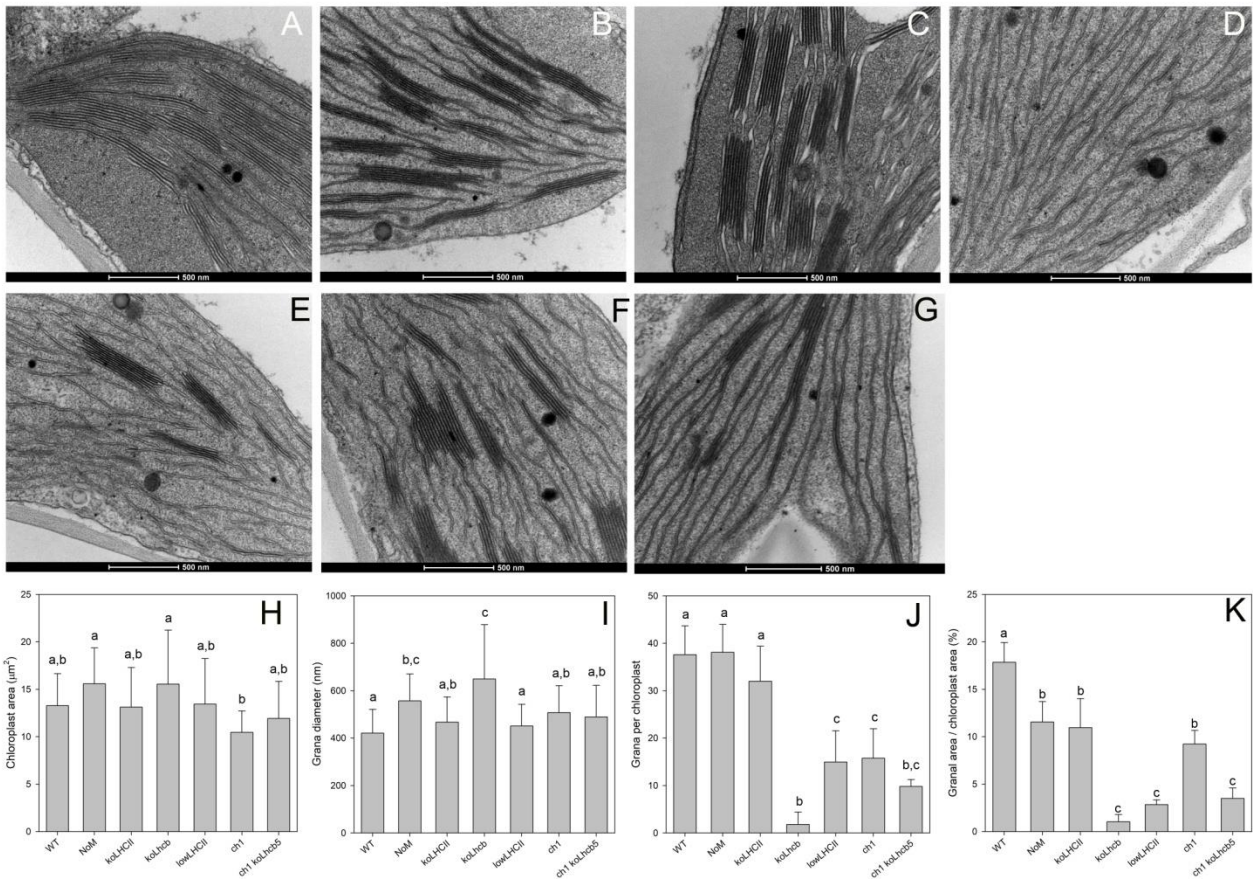
721 **Figure 1. Phenotype of wild type and mutant plants. (A)** Plants were grown for 6 weeks at 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 23  $^{\circ}\text{C}$ , 8/16 h light/dark (LL). **(B)** The fresh weight of all. Values are expressed as mean  $\pm$  SD, n=10. **(C)** Leaf Chl content. *koLhcb* lines retained only 25% of Chl per area, while *NoM* and *koLHCII* retained 75% and 50%, respectively. Values are expressed as mean  $\pm$  SD, n=4. **(D)** Plant growth for 6 weeks at 350  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 23  $^{\circ}\text{C}$ , 8/16 h light/dark (HL). *NoM* and *koLHCII* showed similar growth while *koLhcb* lines were much smaller, alike in CTRL light (panel B). Leaf Chl content relative to the wild type remained essentially the same in LL vs HL (panel C). Values that are significantly different from the corresponding wild type (ANOVA followed by Tukey's post-hoc test at a significance level of  $P < 0.05$ ) are marked with different letters.

722  
723  
724  
725  
726  
727  
728



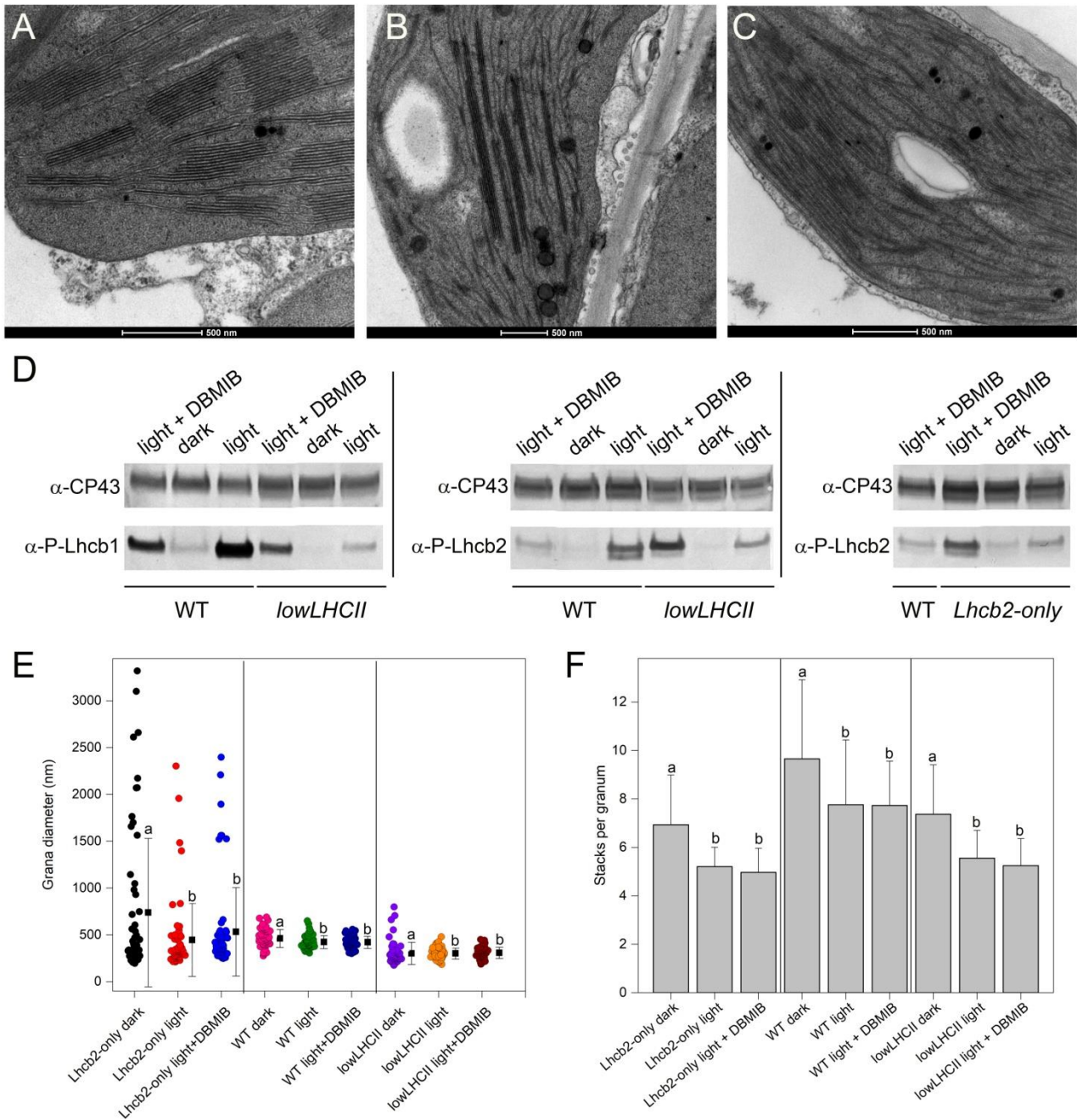
729

730 **Figure 2. Biochemical and functional characterization of the photosynthetic apparatus of wild type and**  
 731 **mutant plants. (A)** Non-denaturing Deriphat-PAGE of thylakoids upon solubilization with 0.8%  $\alpha$ -DM,  
 732 revealing pigment-protein complexes of wild type, *NoM*, *koLHCII* and *koLhcb* lines. Thylakoid proteins  
 733 corresponding 20-35  $\mu$ g of Chls were loaded in each lane. The composition of major bands is indicated based  
 734 on previous reports, while that of bands BD1-BD4 was determined from absorption spectra and SDS-PAGE  
 735 (Supplementary Figure S2). **(B)** PSII functional antenna size of wild type and mutants, measured at RT on  
 736 leaves vacuum-infiltrated with 50  $\mu$ M DCMU. The reciprocal of time corresponding to two-thirds of the  
 737 fluorescence rise ( $t_{2/3}^{-1}$ ) was taken as a measure of the PSII functional antenna size. Plants were dark-adapted  
 738 for 30 min before measurements. Data are expressed as mean  $\pm$  SD ( $n=9$ ). Values marked with different  
 739 letters are significantly different from each other (ANOVA followed by Tukey's post-hoc test at a significance  
 740 level of  $P < 0.05$ ). **(C)** PSII maximal quantum yield ( $F_v/F_m$ ) of wild type, *NoM*, *koLHCII* and *koLhcb* lines,  
 741 measured on dark adapted leaves. Symbols and error bars show means  $\pm$  SD ( $n=4$ ). Values that are  
 742 significantly different (ANOVA followed by Tukey's post-hoc test at a significance level of  $P < 0.05$ ) are marked  
 743 with different letters.



744

745 **Figure 3. Transmission electron micrographs of plastids from leaf mesophyll cells of wild type and mutant**  
 746 **lines. (A-D)** Plants grown in short-day conditions were dark-adapted for 2 hours before harvesting leaves,  
 747 then samples were fixed, embedded, and observed in thin sections at different levels of magnification. Wild  
 748 type (A), *NoM* (B) and *koLHCII* (C) showed a characteristic organization of stroma lamellae interconnecting  
 749 grana, while the chloroplasts of *koLhcb* (D) lacked grana. **(E-G)** Micrograph of *lowLHCII* (E), *ch1* (F) and *ch1*  
 750 *koLhcb5* (G) chloroplasts. **(H-K)** Statistical analysis of the extent of the thylakoid stacking in the wild type, LHC  
 751 KO lines and Chl *b*-less mutants *ch1* and *ch1 koLhcb5*. Histograms report: (H) chloroplast area ( $n \geq 12$ ); (I) grana  
 752 diameter [ $n = 22$  (wild type), 27 (*NoM*), 26 (*koLHCII*, *Lhcb2-only*), 7 (*koLhcb*), 25 (*ch1* lines)]; (J) grana per  
 753 chloroplast ( $n \geq 8$ ); (K) granal area vs. chloroplast area ( $n \geq 5$ ). Data are shown as mean  $\pm$  SD. Values that are  
 754 significantly different (ANOVA followed by Tukey's post hoc test at a significance level of  $P < 0.05$ ) are marked  
 755 with different letters.



756

757

758

759

760

761

762

763

764

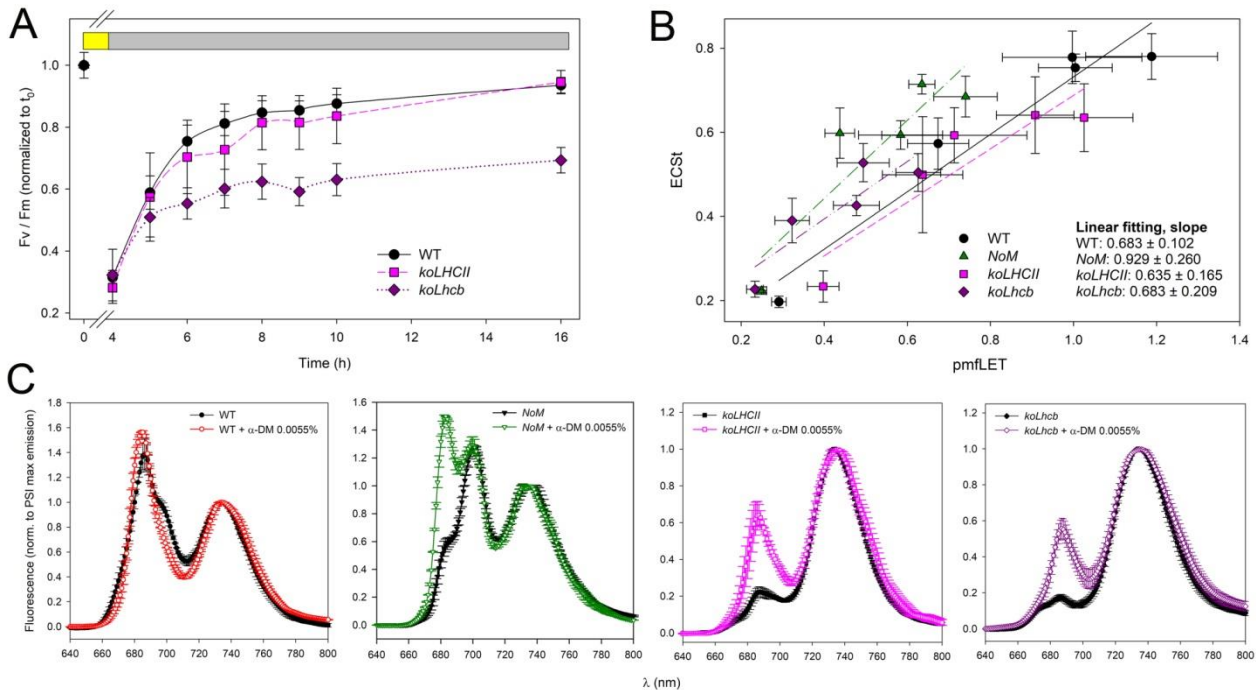
765

766

**Figure 4. Changes in chloroplast ultrastructure of state 1- and state 2-adapted leaves. (A-C)** Leaves from wild type (A), *Lhcb2-only* (B) and *lowLHCII* (C) plants were subjected to 2 hours of PSII light ( $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) to promote LHCII phosphorylation and transition to state 2. Reference samples included leaves either dark-adapted for 1 hours or exposed to PSII light upon vacuum-infiltration with  $100 \mu\text{M}$  DBMIB to maximize PQ reduction thus LHCII phosphorylation. Leaf discs were then fixed, embedded, and observed in thin sections. **(D)** Leaf discs from the same treatments were snap frozen, then the phosphorylation level of Lhcb1/Lhcb2 was quantified by immunotitration, using  $\alpha\text{-Lhcb1-P}$  and  $\alpha\text{-Lhcb2-P}$  primary antibodies. Proteins corresponding to  $0.8 \mu\text{g}$  of Chls (wild type sample) or  $2.4 \mu\text{g}$  of Chls (mutant samples) were loaded for each sample. All samples were loaded on the same SDS-PAGE slab gel. **(E)** Statistical analysis of differences in grana diameter, in state 1 and state 2 [ $n = 60$  (*Lhcb2-only*),  $55$  (wild type),  $58$  (*lowLHCII*)]. **(F)** Statistical analysis of

767 the average number of layers in grana stacks, in state 1 and state 2 (n = 30). Data (E, F) are expressed as mean  
 768  $\pm$  SD. Values that are significantly different (ANOVA followed by Tukey's post-hoc test at a significance level  
 769 of  $P < 0.05$ ) are marked with different letters.

770



771

772 **Figure 5. Biophysical characterization of the mechanisms regulating energy transduction reactions in**  
 773 **thylakoids. (A)** PSII repair efficiency. Detached leaves were exposed to EL for 3 hours (wild type, 1100  $\mu\text{mol}$   
 774  $\text{photons m}^{-2} \text{s}^{-1}$ ; *koLHCII*, 900  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; *koLhcb*, 750  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 4°C, to reduce PSII  
 775 maximal quantum yield ( $F_v/F_m$ ) to about 30% of the initial value. Recovery of  $F_v/F_m$  was performed at 15  $\mu\text{mol}$   
 776  $\text{photons m}^{-2} \text{s}^{-1}$ , 24°C. *koLhcb* line showed a slower PSII repair kinetic compared to both wild type and *koLHCII*.  
 777 Values are expressed as mean  $\pm$  SD, n = 5. **(B)** Relationship between pmfLET and ECSt in wild type and mutant  
 778 lines upon steady state illumination. All genotypes showed similar amplitude of total ECS as a function of LET,  
 779 thus suggesting a similar amplitude of CEF. Values are expressed as mean  $\pm$  SD, n = 4. **(C)** 77K fluorescence  
 780 emission spectra were recorded on thylakoids from wild type, *NoM*, *koLHCII* and *koLhcb* plants, before and  
 781 after treatment with 0.0055%  $\alpha$ -DM, severing weak interactions between Chl-binding complexes without  
 782 solubilizing the membranes. Upon treatment, wild type and *NoM* showed an increased emission by PSII,  
 783 ascribed to LHCII population disconnected from PSII RC; *koLHCII* and *koLhcb* showed a far higher emission by  
 784 PSII upon treatment, suggesting the presence of PSII-to-PSI spill-over in these lines. Spectra were normalized  
 785 to the maximal emission of PSI-LHCI at 733 nm.  $\lambda_{\text{exc}} = 440$  nm. Symbols and error bars show means  $\pm$  SD, n =  
 786 4. See Supplementary Figure S4 for additional details.

787 **Tables**

788 **Table 1. Pigment content and fluorescence induction parameters determined for leaves of *Arabidopsis* WT**  
789 **and mutant lines.** At least five different plants were tested for each line. Chl/Car, molar ratio between  
790 chlorophylls (*a + b*) and carotenoids. Fresh weight refers to growth after 6 weeks under control conditions.  
791 Data are expressed as mean  $\pm$  SD, *n* = 6 biologically independent leaves. Values marked with different letters  
792 are significantly different from each other within the column (ANOVA, followed by Tukey's post-hoc test at a  
793 significance level of *P* < 0.05). Experiments were repeated independently twice, with similar results.

794

	Chl a / b	Chl / Car	$\mu\text{g Chl cm}^{-2}$	$F_v/F_m$
<b>WT</b>	3.51 $\pm$ 0.09 <sup>a</sup>	3.69 $\pm$ 0.21 <sup>a</sup>	19.4 $\pm$ 0.5 <sup>a</sup>	0.82 $\pm$ 0.01 <sup>a</sup>
<b>NoM</b>	3.28 $\pm$ 0.05 <sup>a</sup>	3.45 $\pm$ 0.09 <sup>a,b</sup>	15.3 $\pm$ 0.74 <sup>b</sup>	0.61 $\pm$ 0.01 <sup>b</sup>
<b>koLHCII #17</b>	5.23 $\pm$ 0.11 <sup>b</sup>	3.37 $\pm$ 0.06 <sup>b</sup>	11.46 $\pm$ 0.55 <sup>c</sup>	0.76 $\pm$ 0.01 <sup>c</sup>
<b>koLHCII #36</b>	5.15 $\pm$ 0.10 <sup>b</sup>	3.34 $\pm$ 0.02 <sup>b</sup>	11.03 $\pm$ 0.58 <sup>c</sup>	0.76 $\pm$ 0.01 <sup>c</sup>
<b>koLhcb #9</b>	6.28 $\pm$ 0.15 <sup>c</sup>	3.12 $\pm$ 0.05 <sup>c</sup>	5.68 $\pm$ 0.15 <sup>d</sup>	0.50 $\pm$ 0.02 <sup>e</sup>
<b>koLhcb #16</b>	6.14 $\pm$ 0.22 <sup>c</sup>	3.09 $\pm$ 0.08 <sup>c</sup>	6.10 $\pm$ 0.76 <sup>d</sup>	0.54 $\pm$ 0.03 <sup>e</sup>
<b>ch1</b>	-	2.77 $\pm$ 0.02 <sup>c</sup>	6.55 $\pm$ 0.54 <sup>d</sup>	0.78 $\pm$ 0.01 <sup>c</sup>
<b>ch1 koLhcb5</b>	-	2.57 $\pm$ 0.02 <sup>c</sup>	4.50 $\pm$ 0.23 <sup>e</sup>	0.67 $\pm$ 0.01 <sup>d</sup>
<b>lowLHCII #14</b>	4.01 $\pm$ 0.22 <sup>d</sup>	3.01 $\pm$ 0.06 <sup>b</sup>	7.24 $\pm$ 0.87 <sup>c</sup>	0.52 $\pm$ 0.05 <sup>e</sup>
<b>lowLHCII #32</b>	4.71 $\pm$ 0.39 <sup>b,d</sup>	2.98 $\pm$ 0.03 <sup>b</sup>	7.58 $\pm$ 1.01 <sup>c</sup>	0.55 $\pm$ 0.05 <sup>e</sup>
<b>Lhcb2-only</b>	3.96 $\pm$ 0.16 <sup>e</sup>	3.18 $\pm$ 0.11 <sup>b</sup>	9.77 $\pm$ 1.82 <sup>c</sup>	0.51 $\pm$ 0.02 <sup>e</sup>

795

796

797

798

799

800

801

802

803

804

805

806

807

808

809

