An intact light harvesting complex I antenna system is required for complete state transitions in Arabidopsis

Samuel L. Benson1†, Pratheesh Maheswaran2†, Maxwell A. Ware2, C. Neil Hunter1, Peter Horton1, Stefan Jansson2, Alexander V. Ruban2 and Matthew P. Johnson1*  

Efficient photosynthesis depends on maintaining a balance between the rate of light-driven electron transport occurring in the reaction centres of the two photosystems (PSI and PSII) located in the chloroplast thylakoid membranes. Balance is achieved through a process of ‘state transitions’ that increases energy transfer towards PSI when PSII is overexcited (State II), and towards PSII when PSI is overexcited (State I). This is achieved through redox control of the phosphorylation state of light-harvesting antenna complex II (LHCII). PSI is served by both LHCII and four light-harvesting antenna complex I (LHCI) subunits, Lhca1, 2, 3 and 4. Here we demonstrate that despite unchanged levels of LHCII phosphorylation, absence of specific Lhca subunits reduces state transitions in Arabidopsis. The severest phenotype—observed in a mutant lacking Lhca4 (ΔLhca4)—has a 69% reduction compared with the wild type. The amounts of the PSI–LHCl–LHCII supercomplex isolated by blue native polyacrylamide gel electrophoresis (BN-PAGE) from digitonin-solubilized thylakoids were similar in the wild type and ΔLhca4 mutants. Fluorescence excitation spectroscopy revealed that in the wild type this PSI–LHCII–LHCII supercomplex is supplemented by energy transfer from additional LHCII trimers in State II, whose binding is sensitive to diginiton, and which are absent in ΔLhca4. The grana margins of the thylakoid membrane were found to be the primary site of interaction between this ‘extra’ LHCII and the PSI–LHCII–LHCII supercomplex in State II. The results suggest that the LHCI complexes mediate energetic interactions between ‘extra’ LHCII and PSI in the intact membrane.

Plants regulate the light-harvesting antenna size of PSI and PSII to balance the rate of photochemistry of each reaction centre and so optimize photosynthetic electron transport in response to light quantity and spectral quality1,2. Known as state transitions, this mechanism is controlled by the redox state of the intersystem electron carrier plastoquinone (PQ)3. When the PQ pool is reduced the membrane-associated STN7 serine-threonine kinase begins to phosphorylate the Lhcb1 and Lhcb2 subunits of the major trimeric LHCII (ref. 4). Phosphorylation of LHCII leads to its dissociation from PSII and association with PSI (State II), rebalancing the input of excitation energy between the photosystems over several minutes. When the PQ pool becomes oxidized the STN7 kinase is deactivated and the constitutively active TAP38/PpH1 phosphatase dephosphorylates LHCII leading to its re-association with PSII (State I)5,6. The absence of state transitions in ΔSTN7 Arabidopsis significantly reduces their growth rate under fluctuating light conditions, highlighting the importance of this regulatory mechanism for plant fitness7,8.

PSII and PSI are segregated in thylakoid membranes, with the former residing mainly in the stacked grana regions and the latter in the unstacked stromal lamellae and grana margin regions9. LHCII binds to PSII at three sites named S (strong), M (medium) and L (loose)10. PSI assembles in vivo as a dimer binding two copies each of the M and S trimers, forming the C5S2M2 supercomplex10. The S trimer is formed from Lhcb1 and 2 subunits, while the M trimer comprises Lhcb1 and Lhcb311. Since the structure of the PSII–LHCl–LHCII supercomplex is unchanged following phosphorylation12 and given PSI binds no Lhcb3 in State II (ref. 11), the S and M trimers are unlikely to be involved in state transitions. Therefore, the peripherally bound ‘L’ trimers were suggested to interact with PSI in State II (ref. 11). Electron microscopy has shown that LHCII phosphorylation leads to a partial reduction in the number of layers and lateral dimensions of the grana stacks13,14. The changes in membrane structure result in enrichment of LHCII in the stromal lamellae, and PSI in the grana margins, facilitating increased contact between the two complexes in State II13,15–17.

A PSI–LHCII supercomplex is formed in State II, which depends on the presence of the Psal and Psah subunits of PSI that form the docking site for LHCII11,18–20.

On the opposite side of the PSI complex from the Psal/L binding site of LHCII and not believed to be involved in state transitions are the four LHCl subunits, Lhca1, 2, 3 and 4 (refs. 21–23). Each Lhca subunit shows a 1:1 stoichiometry with PSI, forming a PSI–LHCII supercomplex24–26. Lhca1 and 4 form a dimeric LHCl complex closest to the Psal subunit, while Lhca2 and 3 form a second dimer binding closest to Psal (ref. 26). Absence of Lhca2 or 4 also resulted in a strong reduction of the amount of their dimeric partners, in both the PSI–LHCII supercomplex24,26 and thylakoids27–30. In contrast, loss of Lhca1 or Lhca3 did not destabilize Lhca2 and 4 binding to the same extent28. In the following study we investigated how loss

1Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK. 2School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK. 3Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, Umeå SE-901 87, Sweden. 4Present addresses: Rothamsted Research, West Common, Harpenden, Hertfordshire AL5 2JQ, UK (S.L.B.); Allied Health Sciences Unit, Faculty of Medicine, University of Jaffna, Aadiyapatham Road, Kokuvil East, Kokuvil, Sri Lanka (P.M).
5†E-mail: matt.johnson@sheffield.ac.uk
of the Lhca 1, 2, 3 and 4 subunits affects state transitions in Arabidopsis. The results demonstrate an unexpected role for LHCl in energetically connecting LHCCI to PSI in State II.

Results

Loss of Lhca subunits impairs state transitions. State transitions can be measured by pulse amplitude modulated (PAM) chlorophyll fluorescence, using red (655 nm) and far-red (720 nm) light to preferentially excite PSII and PSI respectively.1,2 Figure 1 compares fluorescence traces for the wild type and mutants (ΔLhca1, ΔLhca2, ΔLhca3 and ΔLhca4). The first part of the trace in which plants were illuminated with both red and far-red light shows the transients associated with the activation of electron transport and the Calvin cycle. After 5 min the far-red light is switched off causing a rapid rise in the fluorescence level (Fs). The Fs rise results from less efficient quenching of the PSI antenna by photochemistry due to over-reduction of the PQ pool. Throughout the subsequent 20 min in the wild type, Fs gradually returns to a value similar but not identical to that seen before the far-red light was switched-off (Fig. 1a). The fall in Fs is related to the state transition, which increases the photochemical rate of PSI, thus re-oxidizing the PQ pool. A saturating light pulse is then applied to different Fm’II (the maximum level of fluorescence in State II). Following this cycle the far-red light is reapplied, and Fs rises gradually over 20 min as LHCCI is dephosphorylated and reconnected to PSI. A second saturating pulse is then applied to determine Fm’I. The mutants displayed a larger rise in Fs upon removal of far-red light and a slower and less complete state transition (Fig. 1b–f). The severest state transition phenotype (measured by the qT method) is seen in ΔLhca4, which has just 31% of the wild-type level, compared with around 52–63% in ΔLhca1, 2 and 3 (Table 1). Consistent with the disruption to state transitions, an increased reduction of the PQ pool is also observed in the mutants (Table 1).

Absence of Lhca subunits does not affect LHCCI phosphorylation.

We tested the phosphorylation state of the thylakoid proteins in State I or State II light conditions using the Diamond Pro-Q Phospho stain. Both the wild type and mutants showed increased phosphorylation of LHCCI and the PSI core subunits D1, D2 and CP43 in State II (Fig. 1g). However, there was no significant difference in the level of LHCCI phosphorylation between the wild type and mutants in either State I or II (Fig. 1g and Supplementary Fig. 1a). Similar results were obtained by anti-phosphothreonine antibody immunoblotting (Supplementary Fig. 1b). These results imply that the deficiency in state transitions is not due to altered activity of the STN7 kinase or PPHP1/TAP38 phosphatase.

Loss of Lhca subunits impairs energy transition from LHCCI to PSI.

The antenna size of PSI in the wild type and mutants was determined by measuring the photo-oxidation kinetics of the PSI reaction centre (P700) using absorption spectroscopy (Fig. 2a,b). Fitting the curves with monoeponential functions showed the PSI antenna size in State I was 84, 72, 82 and 55% of the wild type in ΔLhca1, 2, 3 and 4 respectively (Fig. 2a and Table 2). In State II we calculated an increase of 28% in PSI antenna size in the wild type, consistent with the 25–33% increase previously reported18 (Fig. 2b and Table 2). If state transitions were unperturbed in the mutants, a larger percentage increase in antenna size in State II would be predicted, given their smaller antenna size in State I. In contrast, the PSI antenna size in ΔLhca1, 2, 3 and 4 was increased only by 19, 17, 16 and 17% relative to the State I situation in each sample. The P700’ absorption data therefore shows that the absolute amount of LHCCI energetically coupled to PSI in State II is reduced.

We also quantified PSI antenna size by low temperature (77 K) fluorescence excitation and emission spectroscopy. In the wild-type thylakoids the fluorescence emission showed a large increase in the ratio of the 730 nm PSI band compared to the 685 nm PSII band (Fig. 2c). The PSI emission in ΔLhca1, 2, 3 and 4 thylakoids was blue-shifted from 730 nm in the wild type to 728, 724, 725 and 722 nm respectively ΔLhca1, 2, 3 and 4 (Fig. 2c). The increase in the intensity of the PSI band relative to the 685 nm PSII band in State II was also much smaller in the mutants (Fig. 2c). The increase in PSI antenna size due to state transitions can be calculated by comparing the excitation spectra for 735 nm emission, where PSI contribution is dominant. The spectra are normalized at 705 nm, the PSI–LHCCI terminal emitter region, where there is no absorption by PSI or LHCCI (refs 2,20) (Fig. 2d). A 32% increase in PSI antenna size, expressed as the ratio between the difference spectrum and State I spectrum, was calculated for the wild type. The PSI excitation spectra of the mutants showed differences with the wild type, consistent with the selective loss of specific Lhca subunits in each case (Fig. 2d). PSI antenna size increased in State II by 18, 16, 21 and 23% in ΔLhca1, 2, 3 and 4, respectively, confirming the P700’ measurements that show the change is smaller than in the wild type. Nonetheless, the spectral shape of the State II-minus-State I difference spectrum in each case still closely resembled that of the purified LHCCI trimer, confirming that some association between PSI and LHCCI still exists in State II in these mutants (Supplementary Fig. 2a). The changes in PSI antenna size during state transitions were also checked by monitoring the excitation spectra for 705 nm emission, where PSI dominates (Supplementary Fig. 2b). In the wild type the PSI antenna size decreased by 13% in State II, whereas smaller decreases of 9, 8 and 4% were recorded in ΔLhca1, 2, 3 and 4, respectively. Therefore, in the mutants a larger proportion of the LHCCI antenna remains energetically coupled to PSI under State II conditions.

To assess the relative difference in PSI and PSI antenna size, the area under the 705 and 735 nm excitation spectra recorded for State II were normalized to the 685–735 nm emission ratio and then subtracted to give PSI-minus-PSI difference spectra (Fig. 2e). A ΔLhca4 shows the greatest difference between PSI and PSI in the absorption of light at 620–670 nm, consistent with the smaller PSI antenna size in this mutant (Fig. 2e). The relative absorption by PSI of far-red light was also weakest in ΔLhca4, consistent with the loss of some of the far-red absorbing chlorophylls in this mutant (Fig. 2e). When the absorption difference at 635 nm (State II light) is plotted against the 1-qP value of each mutant in State II (Table 1), a negative Q2 linear correlation is observed (Fig. 2f). Therefore the larger differences in the absorption of red light by PSI and PSI results in an increased imbalance in their electron transfer rates and thus increased reduction of the PQ pool in the mutants.

Effect of Lhca on PSI–LHC supercomplex formation. Previous studies of thylakoid membranes solubilized with the detergent digitonin demonstrated formation of a supercomplex comprising PSI–LHCCI and one LHCCI trimer in State II (refs. 11,20). We analyzed thylakoids prepared in State I or State II from the wild type and mutants (Fig. 3a), using a similar protocol of digitonin solubilization and fractionation using BN–PAGE.13 Several bands are resolved in the BN–PAGE gel (Fig. 3a), whose identities were confirmed by denaturing PAGE in the second dimension (Supplementary Fig. 3a) as (from top of the gel to bottom) (1) PSI–LHCCI–LHCII supercomplex, (2) PSI–LHCCI, (3) ATP synthase, (4) PSI core, (5) PSI core, (6) cytochrome bc1 (cytb6f) and (7) trimeric LHCCI. The enrichment in PSI-associated bands relative to PSI in the digitonin solubilized material reflects the composition of the stromal lamellae and grana margins that are accessible to this detergent, while the PSI-enriched grana membranes remain largely unsolubilized15. Consistent with
expectations, in the wild-type State II sample a prominent band belonging to the PSI–LHCI–LHCII supercomplex appears above the PSI–LHCI band that is absent in State I (Fig. 4a).

In State I ΔLhca1 and 3, band 2 migrates slightly further (Fig. 3a), as expected for a PSI–LHCI complex lacking these specific Lhca subunits. A small amount of PSI cores lacking any Lhca subunits (band 4) (Fig. 3a and Supplementary Fig. 3b,c) are also observed in ΔLhca1 and 3, despite being virtually absent in the wild type. Upon transition to State II a new band (band 1) appears above the PSI–LHCI band that is a PSI–LHCI–LHCII supercomplex lacking the respective Lhca subunit in each case (Fig. 3a and Supplementary 3b,c). The situation in ΔLhca2 differs slightly. Firstly, an additional band 2 is observed in State I (labelled band 2b in Fig. 3a) that is the smaller PSI–LHCI complex lacking both Lhca2 and 3, as previously described (Fig. 3a and Supplementary Fig. 3d). In State II band 1 appears but migrates faster than in the wild type and corresponds to a PSI–LHCI–LHCII supercomplex lacking Lhca2 (Fig. 3a and Supplementary Fig. 3d). In ΔLhca4, band 2 is virtually absent but band 2b, representing a PSI–LHCI complex lacking Lhca1 and 4, is present alongside a much larger amount of ‘free’ PSI core complex lacking all Lhca subunits (Fig. 3a and Supplementary Fig. 3e). Upon transition...
to State II, band 1 appears in ΔLhca4, but migrating faster than in the wild type, representing a PSI–LHCI–LHCII supercomplex lacking Lhca1 and 4 (Fig. 3a and Supplementary 3e). Band 2b also increases at the expense of band 4 and this band now contains LHCII, suggesting formation of a PSI core–LHCII supercomplex lacking any Lhca proteins (Fig. 3a and Supplementary Fig. 3e).

Densitometric analysis of the PsaA/B PSI core subunit spot in Sypro-stained 2D gels indicates that 43, 39, 42, 41 and 50% of PSI is present in bands also containing LHCII in the wild type and ΔLhca1, 2, 3 and 4 respectively (Fig. 3b). The biochemical analysis therefore shows that despite the strong reduction in state transitions in the mutants, the amount of PSI complexes binding LHCII, as

Table 1 | Fluorescence parameters of wild-type and ΔLhca mutant plants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fv/Fm</th>
<th>q5</th>
<th>q5 (t½, s)</th>
<th>1-qP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.79 ± 0.02</td>
<td>0.121 ± 0.01</td>
<td>0.81 ± 0.06</td>
<td>144 ± 11</td>
</tr>
<tr>
<td>ΔLhca1</td>
<td>0.82 ± 0.02</td>
<td>0.076 ± 0.02</td>
<td>0.69 ± 0.05</td>
<td>229 ± 22</td>
</tr>
<tr>
<td>ΔLhca2</td>
<td>0.83 ± 0.01</td>
<td>0.064 ± 0.01</td>
<td>0.62 ± 0.05</td>
<td>275 ± 33</td>
</tr>
<tr>
<td>ΔLhca3</td>
<td>0.83 ± 0.01</td>
<td>0.074 ± 0.02</td>
<td>0.66 ± 0.07</td>
<td>245 ± 27</td>
</tr>
<tr>
<td>ΔLhca4</td>
<td>0.82 ± 0.01</td>
<td>0.038 ± 0.01</td>
<td>0.33 ± 0.05</td>
<td>230 ± 25</td>
</tr>
</tbody>
</table>

Fv/Fm, is the maximum quantum yield of PSII in the dark prior to illumination, qT and qS are measures of state transitions, qT is the fluorescence decline associated with movement of LHCII from PSII to PSI and qS measures how effectively state transitions are at rebalancing electron transport. qS(t½) is the half-time taken to reach 50% of the total qS, 1-qP is a measure of the redox state of the intersystem electron carrier plastoquinone in State II.
Table 2 | PSI functional antenna size in wild-type and ΔLhca mutant thylakoids determined by absorption spectroscopy.

<table>
<thead>
<tr>
<th>Sample</th>
<th>State IP700° (t½, ms)</th>
<th>State IIIP700° (t½, ms)</th>
<th>Calculated PSI antenna size % of wild type in State I</th>
<th>% increase in PSI antenna size in State II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>179 ± 5</td>
<td>140 ± 8</td>
<td>100%</td>
<td>28 ± 3%</td>
</tr>
<tr>
<td>ΔLhca1</td>
<td>210 ± 6</td>
<td>174 ± 6</td>
<td>84 ± 3%</td>
<td>19 ± 4%</td>
</tr>
<tr>
<td>ΔLhca2</td>
<td>241 ± 5</td>
<td>212 ± 10</td>
<td>74 ± 3%</td>
<td>16 ± 4%</td>
</tr>
<tr>
<td>ΔLhca3</td>
<td>217 ± 9</td>
<td>185 ± 8</td>
<td>82 ± 3%</td>
<td>17 ± 4%</td>
</tr>
<tr>
<td>ΔLhca4</td>
<td>324 ± 9</td>
<td>278 ± 11</td>
<td>55 ± 5%</td>
<td>17 ± 3%</td>
</tr>
</tbody>
</table>

P700° formation kinetics (830–875 nm) were measured on isolated thylakoid membranes presence of 30 μM DCMU, 100 μM methyl viologen and 500 μM sodium ascorbate to create a donor-limited situation. Traces were fitted with a single exponential functions and the tabulated data is the average of four traces per sample. The light intensity was 29 μmol photons m⁻² s⁻¹. PSI antenna size is calculated as (wild-type State I t½ ÷ sample t½) × 100% expressed as a percentage of wild-type State I.

Figure 3 | Effect of digitonin on PSI–LHCII interaction in wild-type and ΔLhca thylakoids. a, BN–PAGE of supernatant from 1% digitonin solubilization of thylakoid membranes from wild type and ΔLhca mutants. b, Relative amounts of PSI binding LHCII as derived from scanning of the PsaA/B PSI core subunits spot in Sypro-stained 2D gels in Supplementary Fig. 3. c,d, Low temperature (77 K) fluorescence emission spectra (435 nm excitation, spectra were normalized at 685 nm) (c) and PSI low temperature (77 K) fluorescence excitation spectra (735 nm emission spectra were normalized at 705 nm) (d) following 1% digitonin treatment of State II thylakoids (dashed line) compared to untreated State I (solid blue line) and State II (solid red line) wild-type and ΔLhca4 thylakoids.
determined by the Digitonin BN–PAGE method, is similar to the wild type.

We next analyzed whether the increase in PSI antenna size in State II, determined by excitation fluorescence and absorption spectroscopy, could be explained by the amount of PSI binding LHCII observed by the Digitonin BN–PAGE method. Assuming that the in wild-type State I corresponds to the antenna size in the PSI–LHCI complex (155 chlorophylls)\(^{22,23}\), then 55% of this is ∼85 chlorophylls for \(\Delta Lhca4\). If 50% of this binds 42 extra chlorophylls (one LHCII trimer)\(^{32}\) then the increase would be 25%, consistent with the 23% rise observed (Fig. 2d). In contrast, the 32% rise observed in wild-type PSI antenna size in State II would imply an extra ∼1.2 trimers are bound per PSI. Clearly this is not observed by the Digitonin BN–PAGE method where only ∼45% of PSI can be isolated with LHCII bound (Fig. 3b). Therefore a clear discrepancy emerges between the two sets of data.

Lhca proteins mediate energy transfer between the “extra” LHCII and PSI. Recent evidence implies that energy transfer from LHCII to PSI is disrupted by the addition of digitonin to thylakoid membranes\(^{33}\). These data suggested that the stable PSI–LHCI–LHCII supercomplex isolated by the Digitonin BN–PAGE method from State II thylakoids may be supplemented by energy transfer from extra LHCII trimers, whose binding is more sensitive to the presence of digitonin. Indeed, large proportions of ‘free’ LHCII trimers are recovered upon even gentle solubilization of thylakoids with digitonin (see Fig. 3a, band 7). To investigate this further, we followed the approach of Grieco et al.\(^{33}\) adding 1% digitonin to the State II wild-type and \(\Delta Lhca4\) thylakoids and assessing the changes in the 77 K thylakoid fluorescence emission spectrum (Fig. 3c). The total fluorescence emitted by the PSII bands was increased relative to PSI in the presence of digitonin, with a strong shoulder appearing at ∼680 nm that is characteristic for emission from uncoupled LHCII trimers (Fig. 3c). The relative decrease in the PSI emission band in the \(\Delta Lhca4\) mutant was clearly smaller than that seen for the wild type. The result could imply that more LHCII is energetically disconnected from PSI in the wild type than in \(\Delta Lhca4\) by the digitonin treatment. To check this we compared the PSI 77 K fluorescence excitation spectra for the digitonin-treated wild-type and \(\Delta Lhca4\) thylakoids.

Figure 4 | PSI antenna size in wild-type grana and stromal lamellae membranes. a, b, Low temperature (77 K) fluorescence emission spectra (435 nm excitation, spectra were normalized at 685 nm) (a) and PSI low temperature (77 K) fluorescence excitation spectra (735 nm emission spectra were normalized at 705 nm) (b) of isolated wild-type State I (blue) and State II (red) grana and stromal lamellae compared to isolated PSI–LHCI (black) and PSI–LHCI–LHCII (grey) supercomplexes. c, State I-minus-State II excitation difference spectrum for grana (G) and stromal lamellae (SL) and relative differences compared to purified PSI–LHCI. d, P700\(^+\) formation kinetics (830–875 nm) measured by absorption spectroscopy on isolated grana and stromal lamellae.
to those obtained on intact thylakoids (Fig. 3d). The results show that the PSI antenna size is decreased by the digitonin treatment by ~50–60% in the wild type, but no clear decrease could be seen in ΔLhca4 (Fig. 3d). Therefore in the wild type there is a population of LHCCI transferring energy to PSI in State II, whose connectivity is sensitive to digitonin and is absent in ΔLhca4.

‘Extra’ LHCCI interacts with PSI in the grana margins. To investigate the membrane location of the ‘extra’ digitonin-sensitive fraction of LHCCI bound to PSI, we fractionated the wild-type digitonin-solubilized thylakoids by differential centrifugation to obtain grana and stromal lamellae (Supplementary Table 1). These membrane fractions were then analyzed by 77 K fluorescence emission and excitation spectroscopy (Fig. 4a,b). The PSI II bands at 685 and 693 nm dominate the emission spectrum of the grana with a smaller peak at 730 nm, belonging to PSI located in the grana margins (Fig. 4a). The intensity of the 730 nm band in the grana fraction increases upon transition to State II (Fig. 4a). When the excitation spectra for 735 nm PSI emission in the State I and State II grana fractions are compared, a 37% increase in PSI antenna size is observed in State II, slightly larger than the 32% change averaged across the whole thylakoid (Fig. 2d). The emission spectrum of the stromal lamellae is dominated by the PSI band at 732 nm, which increases in intensity slightly in State II relative to the 685 nm PSI band (Fig. 4a). The 735 nm PSI excitation spectra of the stromal lamellae show a smaller 14% increase in PSI antenna size in State II (Fig. 4b). The shape of the State II minus-State I excitation difference spectra for grana and stromal lamellae is consistent with trimeric LHCCI (Fig. 4c). We compared the excitation spectra of purified wild-type PSI–LHCCI and PSI–LHCI–LHCCI complexes to those obtained for the grana and stromal lamellae (Fig. 4b). The PSI antenna size was similar in the isolated PSI–LHCI–LHCCI supercomplex and State II stromal lamellae, but was larger in the grana (Fig. 4b). The State II grana minus PSI–LHCI–LHCCI supercomplex difference spectrum demonstrated that this was due to binding of ‘extra’ LHCCI (Fig. 4c). Surprisingly, in State I the PSI antenna sizes in stromal lamellae and grana were both larger than that of the isolated PSI–LHCCI complex (Fig. 4b). In the case of the State I grana, the spectrum more closely resembled that of the PSI–LHCI–LHCCI supercomplex (Fig. 4b). Again, the excitation difference spectra showed features consistent with LHCCI, though they were slightly red-shifted, which may be due to the slight blue-shift of the Qy maximum in the purified PSI–LHCCI complex (Fig. 4c). The excitation data therefore suggest that PSI in the grana margins receives more than 1 LHCCI trimer per PSI as described by the current model of state transitions. Moreover, it seems that some LHCCI still transfers energy to PSI in the virtual absence of LHCCI phosphorylation. Finally, we checked the PSI antenna size in the grana and stromal lamellae compared to purified PSI–LHCCI by P700* absorption spectroscopy (Fig. 4d). The PSI antenna size was found to be 116% and 157% in States I and II in the grana and 107% and 116% in the stromal lamellae compared with the purified PSI–LHCCI complex (Supplementary Table 1), confirming the conclusions drawn from the excitation spectroscopy.

Discussion

In this work we uncovered an unexpected role for the LHCCI antenna system in energetically connecting LHCCI to PSI. The mutants showed a clear reduction in the amount of state transitions compared with the wild type, with the largest decrease seen in ΔLhca4. The state transitions phenotype may explain the strongly reduced Darwinian fitness of ΔLhca mutants, particularly ΔLhca4, observed in field experiments. We note the absence of PsaG, and PsaK PSI core subunits, which bind on the LHCCI side of the PSI–LHCCI, have previously been found to decrease state transitions by around ~30% and ~60% in the mutants, while the levels of Lhca subunits are somewhat reduced in the absence of PsaG and PsaK (refs 34,35). As such, we can conclude the reduced state transition phenotype in these mutants is caused by the absence of Lhca subunits. It is noteworthy that the absence of PsaH or PsaL reduces state transitions by ~60–70%, suggesting that some LHCCI is still able to transfer energy to PSI via another route in these mutants. In light of our results, it appears that this alternative pathway involves the Lhca subunits.

We found the PSI antenna size in the mutants was reduced in both State I and State II compared with the wild type. Impairment of state transitions meant larger differences in the efficiency of red light (620–670 nm) absorption by PSI and PSI exist in the mutants, particularly in State II. The result is an increase in the reduction of the PQ pool in the mutants under red light illumination, indicative of a greater imbalance in photochemical rate between the photosystems. Despite the state transition phenotype we found that the levels of LHCCI and PSI phosphorylation were unperturbed in these mutants. In addition, the PSI–LHCL–LHCCI supercomplex could still be isolated in similar yields from the mutants and the wild type, ~40–50% of the total solubilized PSI. This result led us to investigate the extent to which the PSI antenna size in vivo detected by absorption and excitation fluorescence spectroscopy matched the amount of PSI–LHCL–LHCCI supercomplex isolated by the Digitonin BN–PAGE method. Intriguingly, the amount of PSI binding LHCCI in ΔLhca4 matched the in vivo changes in antenna size, while in the wild type it did not. We thus investigated whether solubilization with 1% digitonin would affect the PSI antenna size measured by fluorescence excitation spectroscopy. In the wild type the PSI antenna size was reduced by ~50–60% due the digitonin treatment, but in ΔLhca4 the PSI antenna size was almost unchanged. These data suggest that in addition to the digitonin-insensitive PSI–LHCL–LHCCI supercomplex, there is an ‘extra’ pool of LHCCI trimers transferring energy to PSI in State II. Energy transfer from this extra LHCCI to PSI is sensitive to the presence of digitonin and is largely absent in ΔLhca4. These results help explain earlier observations that the LHCCI could affect the energy transfer from LHCCI to PSI in membrane preparations from spinach.

The increase in PSI antenna size occurring during state transitions is seen primarily in the grana margins (+37%), with a smaller increase observed for the stromal lamellae (+14%), consistent with the reports of Tikkanen et al. and Kim et al. The results showed the PSI antenna size in the grana margins was even larger than that seen in the isolated PSI–LHCL–LHCCI supercomplex, supporting the notion that extra LHCCI trimers are bound to PSI in vivo. Remarkably, our results demonstrate that even in the virtual absence of LHCCI phosphorylation in State I, the PSI antenna size in vivo is still elevated compared with purified PSI–LHCCI, due to its interaction with trimeric LHCCI. The P700* data allow us to estimate the absolute antenna size by assuming the purified PSI–LHCCI complex corresponds to 155 chlorophylls. Thus the PSI antenna size in the grana is ~180 chlorophylls (0.6 LHCCI trimers/PSI) in State I and ~243 (2.09 trimers) in State II and in the stromal lamellae is ~166 (0.26 trimers) in State I and ~180 (0.6 trimers) in State II. Consistent with this suggestion, a detergent-free membrane preparation of PSI–LHCCI binding up to five LHCCI trimers was recently isolated from spinach using styrene-maleic acid copolymer. Indeed, there are many previous reports in spinach and pea showing that PSI can be isolated with significant quantities of LHCCI (refs 36,39–41) and that PSI in the grana margins has a larger antenna size due to binding of extra LHCCI (refs 9,40,41).

It has been suggested that the large amount of LHCCI trimers, which are recovered upon detergent solubilization of the Arabidopsis thylakoid membrane, unrelated to either PSI or PSII, form an antenna trimers.
The 705 nm excitation spectra were normalized according to the change in the 685/735 nm emission ratio as described previously. When assessing the effect of digitonin on thylakoid fluorescence (Fig. 4a), 0.5 mg/ml (chlorophyll) of thylakoids was solubilized with 0.01% digitonin for 20 min at room temperature and then diluted to 1 µM chlorophyll using fluorescence buffer supplemented with 0.06% digitonin and immediately frozen for measurement.

Received 11 May 2015; accepted 14 October 2015; published xx xx 2014

References

47 antenna of Photosystem I
46 complex.
45 excitation energy transfer between LHCII and LHCI in a chlorophyll-protein
43 thylakoids.
41 photosystem I-light harvesting chlorophyll II membranes from spinach
37 during state transitions in granal and stroma-exposed thylakoid membrane of
32 photosynthetic electron
30 35. Varotto, C.,
29 24701
28 I and the photosystem I reaction center core.
26 34. Jensen, P. E., Gilpin, M., Knoetzel, J. & Scheller, H. V. The PSI-K subunit of
25 607
24 including both photosystems II and I.
22 32. Liu, Z. F.,
21 complexes.
20 30. Ihalainen, J. A.,
19 for separation of thylakoid protein complexes: novel super- and mega-
18 complex protein important for plant
16 photosystem I is involved in the interaction between light-harvesting complex
13 280,
12 chlorophyll
11 photosystem I antenna protein Lhca4 in
9 1525
8 photosystem I.
7 27. Zhang, H., Goodman, H. M. & Jansson, S. Antisense inhibition of the
6 photosystem I antenna system to photosystem I in higher plants. J. Biol.
5 of Lhca complexes in the supramolecular organization of higher plant
3 association of the antenna system to photosystem I in higher plants.
2 25. Morosinotto, T., Ballotarri, M., Klimmek, F., Jansson, S. & Bassi, R. The
1 Q3
Journal: NPLANTS
Article ID: npplants.2015.176
Article Title: An intact light harvesting complex I antenna system is required for complete state transitions in *Arabidopsis*
Author(s): Samuel L. Benson *et al.*

<table>
<thead>
<tr>
<th>Query Nos.</th>
<th>Queries</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Please clarify what the qT method is</td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>Please clarify what the 1-qP value stands for</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>Ref 37: please update the details if available</td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td>Figure 3b: please define the error bars</td>
<td></td>
</tr>
</tbody>
</table>