LETTERS

Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases

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Illumination changes elicit modifications of thylakoid proteins and reorganization of the photosynthetic machinery. This involves, in the short term, phosphorylation of photosystem II (PSII) and light-harvesting (LHCII) proteins. PSII phosphorylation is thought to be relevant for PSII turnover[1,2], whereas LHCII phosphorylation is associated with the relocation of LHCII and the redistribution of excitation energy (state transitions) between photosystems[3,4]. In the long term, imbalances in energy distribution between photosystems are counteracted by adjusting photosystem stoichiometry[5,6]. In the green alga *Chlamydomonas* and the plant *Arabidopsis*, state transitions require the orthologous protein kinases STT7 and STN7, respectively[7,8]. Here we show that in *Arabidopsis* a second protein kinase, STN8, is required for the quantitative phosphorylation of PSII core proteins. However, PSII activity under high-intensity light is affected only slightly in *stn8* mutants, and D1 turnover is indistinguishable from the wild type, implying that reversible protein phosphorylation is not essential for PSII repair. Acclimation to changes in light quality is defective in *stn7* but not in *stn8* mutants, indicating that short-term and long-term photosynthetic adaptations are coupled. Therefore the phosphorylation of LHCII, or of an unknown substrate of STN7, is also crucial for the control of photosynthetic gene expression.

STT7 and STN7 are orthologous protein kinases required for LHCII phosphorylation and for state transitions in *Chlamydomonas* and *Arabidopsis*, respectively[7,8]. In *Arabidopsis*, another STT7/STN7-like protein (STN8) exists that is not required for state transitions[8]. STN8 is located in the chloroplast, as shown by *in vivo* subcellular localization of its amino-terminal region fused to the dsRED protein and by the import of, and transit peptide removal from, STN8 translated *in vitro* (Fig. 1a, b). Chloroplast subfractionation after import revealed that the protein is associated, like STT7 and STN7, with thylakoids (Fig. 1c) (refs 7, 8).

Insertion mutants for *STN8* and *STN7* were obtained from the Salk collection[9], and for each gene two independent mutant alleles lacking the respective transcript were identified (Supplementary Fig. S1). The *stn7 stn8* double mutant was generated by crossing *stn7* and *stn8* single knockouts and screening the resulting F_2 generation for homozygous double mutants. All mutants were indistinguishable from the wild type with regard to the timing of seed germination and growth rate in the greenhouse (Supplementary Fig. S1). In *stn7* and *stn7 stn8* mutants, a slight decrease in the levels of neoxanthin, lutein and total chlorophyll was found (Supplementary Table S1). These subtle changes can be attributed to a minor decrease in LHCII content, not detectable by polyacrylamide-gel electrophoresis (PAGE) analysis (Supplementary Fig. S2).

Photosynthetic electron flow, measured on the basis of chlorophyll fluorescence, was not altered in the mutants (Supplementary Table S2). State transitions were suppressed in *stn7* and *stn7 stn8* plants (Supplementary Table S2) but were not affected in the *stn8* mutant, confirming previous results[8]. Reversible LHCII phosphorylation, which is associated with state transitions[7], was studied *in vivo* in dark-adapted plants incubated with [^{33}P]orthophosphate and then exposed to different light conditions (Fig. 2a). Under low light, wild-type and *stn8* plants showed a marked increase in LHCII phosphorylation, whereas subsequent exposure to high light decreased the amount of phospho-LHCII. In *stn7* and *stn7 stn8* plants, reversible phosphorylation of LHCII was not detectable, again in agreement with previous results[8]. Determination of LHCII phosphorylation with a phosphothreonine-specific antibody (Fig. 2b), or an *in vitro* assay in conditions under which the LHCII

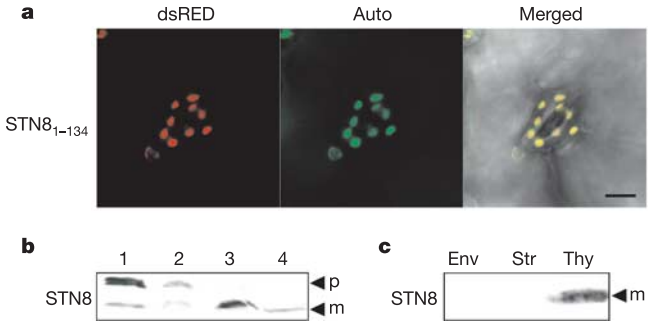

Figure 1 | Subcellular localization of STN8. a, The STN8$_{(1–134)}$–dsRED fusion was stably introduced into *stn8* plants. Guard cells were analysed by confocal laser scanning microscopy. Left, dsRED fluorescence identifying the fusion protein; middle, chloroplasts revealed by chlorophyll autofluorescence (shown in false colour); right, merged images. Scale bar, 50 μm. **b**, ^{35}S-labelled protein, translated *in vitro* (lane 1, 10% translation product) was incubated with isolated chloroplasts for 20 min at 4 °C (lane 2) or 25 °C (lanes 3 and 4), and chloroplasts were recovered by centrifugation through 40% Percoll. Chloroplasts were incubated with thermolysin (lane 4) and subjected to SDS–PAGE; proteins were detected by autoradiography. p, precursor; m, mature protein. **c**, Chloroplasts were fractionated after protein import. Env, envelope; Str, stroma; Thy, thylakoids.

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kinase should be maximally active (Fig. 2c), also showed that a loss of STN7 function, but not of STN8 function, suppresses the phosphorylation of LHCII. These results, together with the spectroscopic data, indicate that STN8 and STN7 do not act in series in phosphorylating LHCII.

Thylakoid protein accumulation was similar in mutant and wild-type plants (Supplementary Fig. S2). When phosphorylation of PSII core proteins was monitored with the phosphothreonine-specific antibody, *stn7* plants behaved like the wild type, showing high levels of phosphorylated PSII core proteins under all light regimes whereas LHCII phosphorylation was greatly decreased (Fig. 2d, e). In contrast, *stn8* plants showed a marked decrease in the total amount of PSII core phosphoproteins—particularly under high light—whereas LHCII phosphorylation was as in the wild type. This again argues that the main substrates of STN7 and STN8 are different: LHCII phosphorylation is mostly dependent on STN7, whereas phosphorylation of PSII core proteins depends almost exclusively on STN8. Strikingly, only in the *stn7 stn8* mutant were the phosphorylated forms of LHCII and the PSII core proteins completely absent under all light regimes tested, indicating that the two kinases must show some degree of overlap in their substrate specificities. However, the clear distinction between the phosphorylation phenotypes of the two mutants implies that STN7 and STN8 act in parallel and could be directly responsible for phosphorylating the LHCII and PSII core proteins, respectively. If, however, the proposal that LHCII is phosphorylated by thylakoid-associated kinase (TAK) proteins[10] is correct, then STN7 might act upstream of TAKs.

Reversible phosphorylation of the D1 protein is thought to have a key function in the regulation of its turnover during the photoinhibition of PSII (ref. 11). Exposure to light induces phosphorylation of, and causes damage to, PSII reaction centres, and the phosphorylated form of damaged D1 is resistant to proteolysis[12]. However, relocation of damaged PSII centres from grana to stroma lamellae permits the dephosphorylation and proteolysis of D1, and co-translational incorporation of newly synthesized D1 (ref. 1). Consequently, lack of the D1 protease impairs the PSII repair cycle[13]. To test whether suppression of PSII core phosphorylation also impairs PSII repair by changing the rate of D1 turnover, we investigated the inactivation of PSII under high light, the recovery of PSII activity and the degradation of D1. Illumination of leaves at high light intensity led to a slightly stronger inactivation of PSII in *stn8* and *stn7 stn8* plants than in *stn7* and wild-type plants; the subsequent recovery of PSII activity under low light was also somewhat slower in *stn8* and *stn7 stn8* than in wild-type and *stn7* plants (Fig. 3a). However, the increased photosensitivity of PSII in *stn8* and *stn7 stn8* plants was not reflected in changes in the rate of light-induced D1 degradation in the presence of lincomycin, an inhibitor of plastid protein synthesis (Supplementary Fig. S3). Moreover, also during photoinhibition and subsequent recovery, no PSII core phosphorylation was detected in *stn7 stn8* plants (Supplementary Fig. S4), excluding the action of another PSII core kinase under these conditions. Pulse–chase experiments under high light intensities

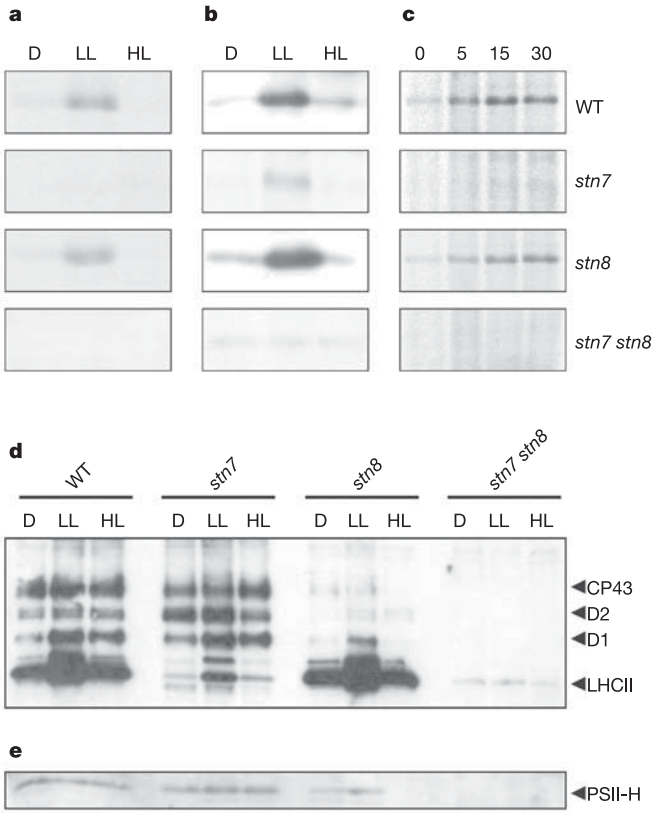

Figure 2 | Phosphorylation of thylakoid proteins. **a**, *In vivo* LHCII phosphorylation. Leaves were incubated with [^{33}P]orthophosphate, and thylakoid proteins from plants kept in the dark (D), subsequently exposed to low light (LL), and then to high light (HL), were fractionated by SDS–PAGE. WT, wild type. **b**, LHCII phosphorylation detected by immunoblot analysis with a phosphothreonine-specific antibody. Leaves were treated and proteins fractionated as in **a**. **c**, *In vitro* LHCII phosphorylation. Thylakoids from dark-adapted leaves were incubated with [γ-^{33}P]ATP under reducing conditions for 0, 5, 15 and 30 min in the dark. Proteins were fractionated as in **a**. **d**, **e**, Phosphorylation of LHCII and PSII core proteins detected by immunoblot analysis. Leaves were treated and thylakoid proteins fractionated as in **b**.

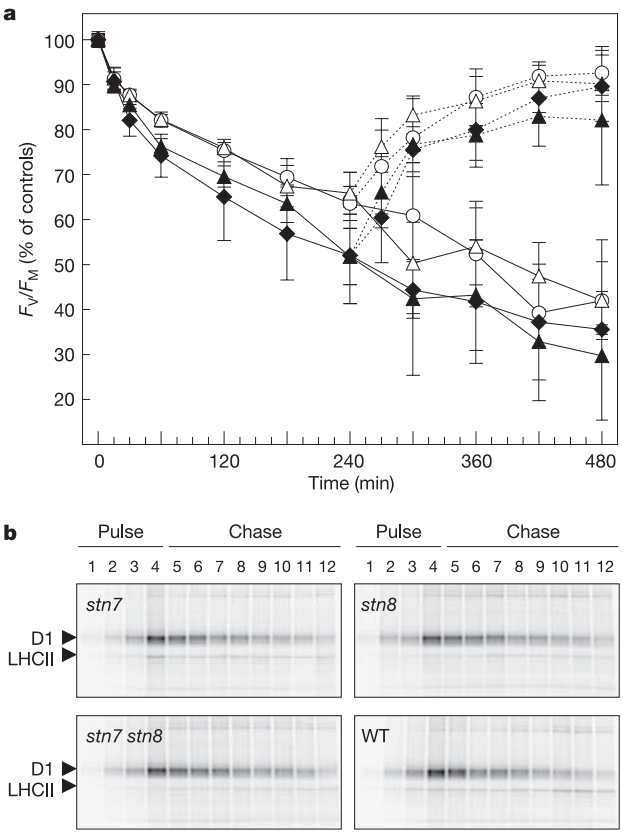

Figure 3 | PSII activity and D1 turnover under high-intensity light. **a**, Time course of PSII inactivation (solid lines) induced by high light (2,000 μmol photons m^{-2} s^{-1}) and recovery under low light (20 μmol photons m^{-2} s^{-1}; dashed lines). Error bars indicate s.d. Open triangles, *stn7*; filled triangles, *stn8*; diamonds, *stn7 stn8*; circles, wild type. **b**, Autoradiogram of thylakoid membrane proteins resolved by SDS–PAGE after pulse-labelling with [^{35}S]methionine for 0, 15, 30 and 60 min (lanes 1–4) and subsequent chase in unlabelled medium for 60, 120, 180, 240, 300, 360, 420 and 480 min (lanes 5–12), both under high light (2,000 μmol photons m^{-2} s^{-1}). WT, wild type.

revealed that rates of D1 synthesis and degradation were similar in all genotypes (Fig. 3b; Supplementary Fig. S5). In addition, wild-type, *stn8* and *stn7 stn8* plants grown under high light intensities behaved very similarly in growth rate and leaf pigment composition (data not shown). Taken together, the data indicate that STN8-mediated phosphorylation of D1 is not crucial for D1 turnover and PSII repair.

Changes in light conditions are thought to result, in the long term, in the adjustment of photosystem stoichiometry, which requires a signalling network that coordinates photosynthetic gene expression in plastids and nucleus[5,6,14]. Recent studies have proposed a functional relationship between LHCII phosphorylation (and state transitions) on the one hand, and the long-term response to altered light conditions on the other[15,16]. The slower growth of *stn7* mutants under fluctuating light has been attributed to impaired state-transition-based adaptation[8]. Here we tested whether defects in the function of STN7 or STN8 affect the changes in antenna structure and photosystem stoichiometry that are associated with the long-term response to changes in the quality of incident light[17,18]. Plants were grown and acclimated to light sources that favoured either PSI or PSII, and the long-term response was followed by monitoring the chlorophyll fluorescence parameter F_S/F_M and the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b*). In the wild type, F_S/F_M values were relatively high after acclimation to PSI light, and low values were measured after acclimation to PSII light, whereas the Chl *a*/*b* ratio behaved in the opposite sense, being high after acclimation to PSII light and low in PSI light[17,18] (Fig. 4a, b). The *stn8* mutant behaved like the wild type (Fig. 4a, b), indicating that its long-term response was normal. In the *stn7* and *stn7 stn8* mutants, however, F_S/F_M and Chl *a*/*b* values were typical of plants acclimated to PSI light under all light regimes tested, indicating that the mutants have lost their capacity for the long-term response and implying that STN7 has a function in coordinating the long-term and short-term responses to changes in light conditions.

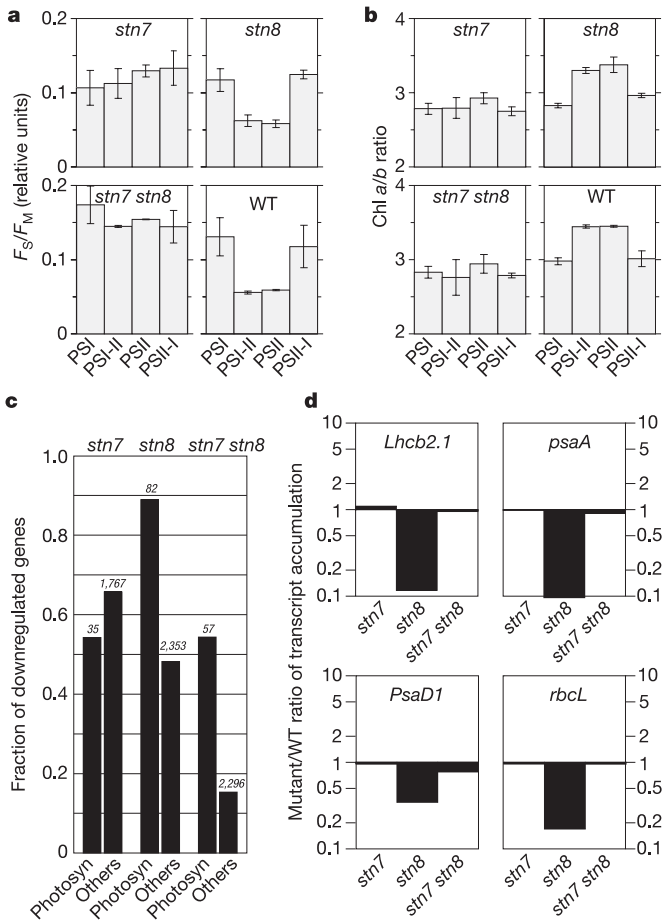

Figure 4 | Photosynthetic acclimation and mRNA expression. a, **b**, Plants were acclimated to PSI or PSII light and F_S/F_M values (**a**) and Chl *a*/*b* ratios (**b**) were determined. Error bars indicate s.d. WT, wild type. **c**, Nuclear transcript accumulation in greenhouse-grown plants. Numbers above bars indicate the total numbers of genes that are significantly differentially expressed in mutants with respect to wild-type plants. Bar lengths indicate the fraction of differentially regulated genes that were downregulated. Photosyn, genes for photosynthesis; others, other genes (for non-photosynthetic proteins). **d**, Mutant versus wild-type ratios of transcript levels (logarithmic scale) of selected nuclear and plastid photosynthetic genes.

Lack of STN7 might impair the long-term response by interfering with a signalling pathway that links changes in photosynthetic efficiency during the adjustment of photosystem stoichiometry to the level of gene expression in plastids[19] and the nucleus[17,18]. To test this we analysed the transcription of photosynthetic genes. For nuclear genes encoding chloroplast proteins two modes of transcriptional regulation are known: a master switch that acts on most genes[20] and an additional mechanism specific for photosynthetic genes[21]. Lack of STN7 in greenhouse-grown *stn7* and *stn7 stn8* plants results in the differential expression of only relatively few photosynthetic genes, in contrast to *stn8* plants in which a large set of photosynthetic genes is markedly downregulated (Fig. 4c; Supplementary Table S3); exemplary expression profiles of two nuclear and two plastid genes are provided in Fig. 4d. The impairment of the transcriptional regulation of certain *stn8*-responsive genes in the absence of STN7, together with the results of the long-term response experiments, therefore argues in favour of a function for STN7 in the regulation of nuclear and plastid gene expression. We can only speculate how STN7 triggers changes in photosynthetic gene expression but, in principle, three hypotheses are available: first, the phosphorylation state of LHCII directly provides information for signalling; second, an unknown protein is phosphorylated by STN7 and participates in signalling; and third, state transitions and the associated conformational changes of thylakoids[3,22] stimulate signalling.

In green algae, the pool of mobile LHCII is large[23], and state transitions are important for cyclic electron flow[3]. Because the mobile LHCII pool in vascular plants is relatively small[24], it is tempting to speculate that triggering of the long-term response, rather than the short-term response in terms of state transitions, represents the major function of STN7 in flowering plants. In this respect, the slower growth of *stn7* plants under fluctuating light[8] might be due to disturbance of transcriptional regulation rather than being a physiological consequence of defects in state transitions. Future analyses must clarify how the short-term and long-term responses are coupled, and whether STN7 and STN8 are necessary and sufficient for the phosphorylation of thylakoid proteins.

METHODS

Plant lines and propagation. Mutant lines from the Salk collection[9] were identified by searching the SiGNAL database (http://signal.salk.edu/tabout.html). Methods for plant propagation have been described elsewhere[25–27].

Chlorophyll fluorescence and pigment analysis. Photosynthetic electron transport, state transitions and leaf pigment composition were measured as described[25–27] (see Supplementary Information for details).

Analysis of LHCII phosphorylation *in vivo* and *in vitro*. To determine the degree of LHCII phosphorylation *in vivo*, dark-adapted leaves from 4-week-old plants were incubated in the presence of [^{33}P]orthophosphate for 1 h and subsequently exposed for 2 h to levels of illumination favouring phosphorylation (low light, 80 μmol photons m^{-2} s^{-1}) or dephosphorylation (high light, 800 μmol photons m^{-2} s^{-1}). Identical amounts of thylakoid proteins (equivalent to 200 mg of fresh leaf) were prepared in the presence of 10 mM NaF[25] and fractionated by SDS–PAGE (14% polyacrylamide); labelled proteins were detected by phosphorimaging (Typhoon; Amersham Biosciences). For the assay *in vitro*, thylakoids isolated from dark-adapted leaves (30 mg of fresh leaf) were incubated with [γ-^{33}P]ATP under reducing conditions in the dark[25]. Separation and detection of thylakoid proteins were performed as described for the *in vivo* assay.

Immunoblot analysis. Leaves from 4-week-old plants were harvested after overnight dark adaptation or light exposure as described above. Thylakoids

were prepared in the presence of 10 mM NaF, fractionated on an SDS–polyacrylamide-gradient gel (8–25% polyacrylamide) and transferred to poly(vinylidene difluoride) membranes[25]. Filters were then probed with antibodies specific for phosphothreonine (Biolabs) and signals were detected by enhanced chemiluminescence (Amersham Biosciences).

Pulse–chase measurement of D1 turnover. The pulse–chase procedure for the analysis of D1 turnover in pea[28] was modified for *Arabidopsis*. For radioactive labelling of thylakoid proteins, leaf discs of 3-week-old *Arabidopsis* plants were pressed extremely gently against coarse sand paper and then vacuum-infiltrated in a syringe containing 1 mCi of L-[^{35}S]methionine in 10 ml of 1 mM KH_2PO_4 pH 6.3, 0.1% Tween 20. Directly after infiltration, three leaf discs were frozen in nitrogen (t_0). Remaining leaves were transferred to high light (2,000 μmol photons $m^{-2}\,s^{-1}$) and for each time point ($t_{pulse} = 15$, 30 and 60 min) three leaf discs were collected. Immediately after the pulse period, the remaining leaf discs were washed, incubated with 10 mM unlabelled L-methionine in the same buffer as before and further exposed to high light for up to 8 h ($t_{chase} = 60$, 120, 180, 240, 300, 360, 420 and 480 min). The three leaf discs for each time point were combined and thylakoid proteins were prepared, separated and detected as described for the LHCII phosphorylation analysis.

***In vitro* import and intracellular localization of dsRED fusions in chloroplasts.** For *in vitro* import assays, ^{35}S-labelled proteins were synthesized and used for import experiments and were detected after subfractionation of chloroplasts[29,30]. For intracellular localization of the dsRED fusion, a complementary DNA fragment coding for the first 134 amino-acid residues of STN8 was fused 5′ to *dsRED* and inserted into the vector pLEELA (Invitrogen), placing it under the transcriptional control of the cauliflower mosaic virus 35S promoter. Seeds were collected from transformed *stn8* plants[26] and independent transgenic plants were selected. Confocal images were collected by laser scanning microscopy (TCS SP2; Leica). Fluorescence was excited with a 461 nm HeNe laser and images were collected in the ranges 565–620 nm (dsRED fluorescence) and 670–750 nm (chlorophyll autofluorescence).

Nucleic acid analysis. *Arabidopsis* DNA was isolated[26] and T-DNA insertion junction sites were recovered by polymerase chain reaction (PCR) with the use of combinations of insertion-specific and gene-specific primers, and then sequenced. To determine levels of gene expression, extraction of total leaf RNA, first-strand cDNA synthesis and reverse-transcriptase-mediated PCR (RT–PCR) were performed[26], using primers specific for *STN7* or *STN8*, as well as *ACTIN1*-specific oligonucleotides as a control.

mRNA expression profiling. Greenhouse-grown mutant and wild-type plants were analysed. The generation and use of a 3292-gene sequence tag (GST) nylon array enriched for nuclear genes for chloroplast proteins, data analysis and statistical evaluation have all been described previously[20,21,26] (see Supplementary Information for details).

Measurement of acclimation to changes in light quality. Light conditions favouring either PSI or PSII (PSI or PSII light, respectively), as well as growth and acclimation conditions for *Arabidopsis*, have been described previously[18]. Plants were initially grown for 10 days under white light followed by a 6-day acclimation period. Seedlings were acclimated either to PSI or PSII light for 6 days or to PSI light for 2 days followed by 4 days under PSII light or vice versa. Measurement of Chl *a*/*b* ratios and determination of the chlorophyll fluorescence parameters F_S (steady-state fluorescence) and F_M (maximal fluorescence), and of F_S/F_M ratios, were performed as described in Supplementary Information. All values under the four conditions were calculated as the means of 50 individuals in at least three independent experiments, and the significance of differences between samples was tested with Student's *t*-test.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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