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State transitions, light-harvesting antenna phosphorylation and light-harvesting antenna migration in vivo in the higher plant *Spirodela oligorrhiza*

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Abstract

Plants can balance the relative levels of excitation energy reaching the two photosystems of photosynthesis via state transitions. This process was investigated in vivo using the aquatic higher plant *Spirodela oligorrhiza*. State transitions were followed by 77 K chlorophyll *a* (Chl *a*) fluorescence and phosphorylation of the Chl *a/b* light harvesting complex (LHCII). A response spectrum for the state transition indicated that light absorbed predominantly by Chl *b* led to state 2 and light absorbed predominantly by Chl *a* resulted in state 1. The kinetics of LHCII phosphorylation ($t_{1/2} = 4$ min) during a state 1 to state 2 transition were similar to the rise in fluorescence at 77 K from Photosystem I (PS I) relative to Photosystem II (PS II) ($t_{1/2} = 3$ min). As well, for the transition from state 2 to state 1, the kinetics of LHCII dephosphorylation ($t_{1/2} = 13$ min) and the rate of loss of fluorescence from PS I relative to PS II ($t_{1/2} = 10$ min) were comparable. The phosphatase inhibitor, NaF, suppressed both LHCII dephosphorylation and the decrease in the PS I/PS II fluorescence emission ratio, thus showing prevention of a transition to state 1 under PS I illumination. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), which indirectly inactivates the LHCII kinase, triggered a transition to state 1 in PS II light. Upon exposure to PS II light, phosphorylated LHCII was observed to appear in granal thylakoid fractions prior to its appearance in stromal thylakoid fractions, indicating vectoral movement between the two membrane compartments. Determinations of the absolute yield of PS II and PS I fluorescence showed a complementary decrease and increase in PS II and PS I emission, respectively, in state 2 compared to state 1. Our results strongly support the hypothesis that LHCII phosphorylation and antenna migration between the photosystems are key components of the state transition mechanism in vivo.

Keywords: Photosystem I; Photosystem II; State 1-state 2 transitions; LHCII kinase; Response spectrum; Protein transport; Thylakoid membranes

1. Introduction

In higher plants the two photosystems of photosynthesis, Photosystem II (PS II) and Photosystem I (PS I), are associated with specific pigment-protein complexes which act as light harvesting antenna [1]. These antenna complexes have distinct absorption characteristics due to the different populations of associated pigment molecules. It is thus possible to change the relative activities of PS II and PS I by changing the

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; F685, fluorescence emission at 685 nm from PS II; F735, fluorescence emission at 735 nm from PS I; LHCII, Chl *a/b* light harvesting complex of PS II; Chl, chlorophyll; PQ, plastoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride.

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wavelength of the actinic illumination. Examples of natural changes in light quality include fluctuations in the angle of the sun and shading by a plant canopy [2].

The imbalanced activities of PS II and PS I can be corrected by regulation of the amount of excitation energy reaching each photosystem. This regulatory mechanism, termed the light state transition, is conveniently described by its two extremes (state 1 and state 2) (for reviews see Refs. [3–7]). The process is readily characterized by changes in room temperature chlorophyll (Chl) fluorescence yield of PS II or by changes in the relative amplitude of PS I and PS II fluorescence at 77 K [4,5,8–14]. State 1 exists when plants are exposed to an excess of light preferentially absorbed by PS I (PS I light; for example, far-red light of 720 nm). In state 1, the plants attempt to direct more of the absorbed light energy to PS II, resulting in increased quantum yield for PS II activity in state 1 relative to state 2 [8,15], as well as increased room temperature and 77 K fluorescence yield from PS II [9–18]. State 2 occurs when light preferentially absorbed by PS II is present (PS II light; for example, red light of 640 nm). This results in distribution of light energy away from PS II, and a concomitant drop in PS II activity and relative fluorescence yield [5,6,9,10,12–15]. It is possible to reversibly convert plants between the two states; for example, by adapting the plants or thylakoid membranes to PS I light and moving them to PS II light, a state 1 to 2 transition will occur [8,12,15].

Numerous reports have connected the state transition process to the reversible phosphorylation of the light-harvesting Chl a/b complex of PS II (LHCII) [5–7,9,10,14,15,17,19]. This group of proteins is strongly phosphorylated by a thylakoid bound kinase under PS II illumination. It was originally thought that when linear electron flow was functioning [20], and thus the plastoquinone pool was reduced [9], the LHCII kinase was activated. However, it was later shown that the LHCII kinase was actually activated due to net reduction of the cytochrome b_6/f complex [21]. Net oxidation of cytochrome b_6/f complexes due to preferential excitation of PS I causes inactivation of the kinase, allowing LHCII to be dephosphorylated by a membrane bound phosphatase activity [9,11,12,19]. It was possible to correlate in vitro phosphorylation of LHCII to the decreased room temperature and 77 K fluorescence yield from PS II that are associated with a state 1 to 2 transition [9–12,14]. Furthermore, the kinetics of a state transition were very similar to the rates of phosphorylation and dephosphorylation of LHCII and it is one of the only thylakoid proteins that is dephosphorylated with the kinetics of a state 2 to 1 transition [12,22,23].

The manner in which phosphorylation of LHCII leads to energy redistribution during state transitions remains elusive. Three theories have been proposed based on LHCII phosphorylation, fluorescence emission and photosynthetic activity data. The first proposes opposite absorption cross-section changes of similar magnitude in the two photosystems that is accomplished by an alteration in the number of antenna complexes associated with each photosystem [6,7,9,12, 13,15,24,25]. During a state 1 to state 2 transition, when LHCII becomes phosphorylated it would freely migrate from PS II to PS I, thus increasing the absorption cross-section of PS I and fluorescence emission from PS I relative to PS II in state 2 [9,10,25–29]. Upon transition from state 1 to 2, dephosphorylation of LHCII would trigger its movement back to PS II. In support of this model, it was shown using 77 K fluorescence emission and an exogenous fluorescence standard that the decrease in PS II fluorescence was accompanied by an increase in fluorescence from PS I [13]. Additional support for this model comes from photoacoustic measurements of oxygen evolution and Emerson enhancement [15]. Furthermore, increased quantum yields of PS I have been observed from thylakoids in state 2 relative to those in state 1 [24,25,30]. However, in conflict with this model, measurements of PS I activity were not always consistent with changes in fluorescence; in some instances the quantum yield of PS I activity did not increase in state 2 [31,32]. Thus, a second theory was proposed in which phospho-LHCII detaches from PS II, but does not associate with PS I [31,32]. The third mechanism suggested that phospho-LHCII becomes associated with a subset of PS II centers (PS II β) [33]. These PS II β-LHCII complexes were hypothesized to associate with PS I increasing the probability of energy transfer from PS II to PS I in a "spillover" mechanism [33,34]. The key concept of the three state transition mechanisms is the redox sensitive phosphorylation of LHCII [9–11,21,22]. Lateral migration in the membrane is another key component of the first and third models. That is, phospho-LHCII from granal thylakoids moves to stromal thylakoids either independently or attached to a PS II β center. Only the second model questions whether phospho-LHCII actually increases the absorption cross-section of PS I in state 2. The one aspect that is the same in the above three mechanisms is a decrease in energy distributed to PS II relative to PS I in state 2.

Much of the work on state transitions in higher plants has been done with in vitro preparations, and some of the above discrepancies could be due to different preparation and incubation protocols. There are few reports of in vivo studies which correlate changes in fluorescence yields with LHCII phosphorylation, and the majority of these were done with algae, detached leaf fragments or leaf disks [15,17,18,21]. We performed a critical study of state transitions using an intact higher plant system to determine which of the processes observed in vitro also occur in vivo. It is

possible to subject whole *Spirodela oligorrhiza* plants (a C-3 monocot) to state transitions by simply altering the light quality, and to monitor 77 K fluorescence in situ or to perform LHCII phosphorylation experiments fully in vivo. As well, inhibitors of phosphorylation and dephosphorylation can be used effectively with the intact plants. Thus, the relationships between the kinetics of fluorescence changes and phosphorylation of LHCII were probed in vivo with intact plants.

2. Materials and methods

2.1. Plant material

Axenic cultures of *Spirodela oligorrhiza* (Kurtz) Hegelm were grown on half-strength Hutner's medium supplemented with 1% sucrose under 20 μmol m^{-2} s^{-1} of visible light. Prior to use, the plants were transferred to medium without sucrose and grown under 50 μmol m^{-2} s^{-1} of continuous visible light in growth chambers supplemented with 3% CO_2 [35]. They were grown under these latter conditions for at least 2 weeks prior to use in state transition assays.

2.2. Induction of state transitions in vivo

Experiments on state transition kinetics were performed by adapting plants to 10 μmol m^{-2} s^{-1} of 640 nm light (PS II light) or 720 nm light (PS I light) for 1 h. Plants were subsequently placed in the opposing light condition (chase light, i.e., the plants adapted to PS I light were moved to PS II light) and the state transition was followed over a 2 h period. For the state 2 response spectrum, plants were taken from visible light and placed in a given wavelength of light for 60 min (all at 10 μmol m^{-2} s^{-1}). Analyses of state transitions were performed by 77 K fluorescence and LHCII phosphorylation as described below.

In some experiments NaF or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were added to half-strength Hutner's medium prior to a state transition. NaF is a phosphatase inhibitor [12,15,19], and DCMU binds to the Q_B site of the D1 protein of PS II resulting in oxidation of the PQ pool [36]. When the plastoquinone (PQ) pool is in an oxidized state, the cytochrome b_6/f complex is also oxidized and this leads to inactivation of the LHCII kinase [21]. Plants were incubated in medium without an inhibitor in either PS I or PS II light for 1 h. The plants were then placed in medium containing either NaF (125 mM) or DCMU (50 μM). The plants were subsequently put in the chase light. In the case of NaF, the inhibitor was added to the medium 10 min prior to incubating in the chase light. Vacuum infiltration was not required for assimilation of either of the chemicals. Samples were analyzed by 77 K fluorescence and LHCII phosphorylation as described below.

2.3. In vivo chlorophyll fluorescence measurements

Detector-corrected low-temperature (77 K) Chl *a* fluorescence emission spectra were obtained with a custom built spectrofluorimeter as described by Bruce et al. [37]. Whole plants were placed as a monolayer on a wooden probe, secured with a glass coverslip and 2 O-rings, and immediately placed in liquid nitrogen. An excitation wavelength of 435 nm was focused on the plant sample held at 77 K in a dewar flask. The leaf tissue was oriented 40° to the excitation beam and fluorescence emission spectra were collected at 90° to the excitation beam with a diode array detector. Data were acquired between 650 and 800 nm and recorded with an IBM-AT compatible computer. Three peaks were detected (Fig. 1); two from PS II at 685 nm and 695 nm (F685 and F695), and one from PS I at 735 nm (F735) [1,37,38]. For most experiments the spectra were normalized to the 685 nm peak and the F735/F685 ratio was used to monitor the progress of state transitions.

It is difficult to obtain consistent fluorescence yields from samples at 77 K. Most of the problems in measurement of both solid and liquid samples in commercial spectrofluorimeters arise from a combination of sample inhomogeneities induced by rapid freezing, light transmission through boiling liquid nitrogen and ice accumulation. The custom built diode array based fluorimeter used in our studies has been optimized for determinations of consistent yield at 77 K, and has been previously shown to give reproducible fluorescence yields (within 4%) from frozen liquid samples [39]. The sample holder was designed so that the actinic light beam was of smaller size than the sample itself. Thus, only a single layer of leaves was irradiated. For one set of experiments in this report, 77 K emission spectra were collected from a number of individual plants which had undergone identical pretreatments and the data were averaged without normalization. For consistency in these experiments, the same sample holder assembly was used to collect all of the individual spectra, so that the variability was limited to the plant material. Due to the consistent pigment content, size, shape and surface texture of Spirodela plants (parameters that did not change during the course of the light treatments employed here) it was possible to obtain highly reproducible fluorescence yields and thus determine absolute changes in 77 K fluorescence emission.

2.4. In vivo LHCII phosphorylation

Whole Spirodela plants were incubated in either PS I or PS II light for 1 h on half-strength Hutner's medium containing [^{32}P]orthophosphate (50 μCi/ml) in place of the non-radioactive orthophosphate. This

level of radioactivity was sufficient to give autoradiogram exposure times of less than 1 week. After the labeling period, plants were placed on new medium without [^{32}P]orthophosphate, put under the chase light and collected at several time points over a 2 h period. Immediately after collection, the plants were frozen in liquid nitrogen and stored in a −80°C freezer until thylakoid membrane proteins were isolated for SDS-PAGE.

2.5. Membrane protein isolation and SDS-PAGE

Thylakoid membrane isolation was performed as described by Marder et al. [40] with a few modifications. Plant tissue (approx. 50 mg) was homogenized in 500 μl of grinding buffer (2.5 mM Tris-HCl (pH 8.5), 50 mM NaCl, 10 mM NaF and 0.1 mM PMSF) using a motor-driven glass/glass homogenizer. The homogenate was separated into soluble and membrane fractions by centrifugation (12000 × *g* for 15 min). The membrane pellet was washed with 300 μl of 2.5 mM Tris/glycine (pH 7.8), 150 mM NaCl, 10 mM NaF, and repelleted at 12000 × *g* for 15 min. The resulting membrane fraction was washed with Tris/glycine, collected by centrifugation (12000 × *g*) and resuspended in 300 μl of sample buffer for SDS-PAGE (62.5 mM Tris-HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 3% (w/v) sodium dodecyl sulfate, 10% (v/v) glycerol). All the above steps were performed at 4°C.

SDS-PAGE, Coomassie blue staining and autoradiography were performed as described by Marder et al. [40]. SDS-polyacrylamide gels were loaded on an approximate equal protein basis (10 μg per lane). After electrophoresis, the gels were stained with Coomassie blue and radiolabel was detected by autoradiography. The radiolabel in LHCII was quantified by scanning densitometry of the autoradiogram (Molecular Dynamics). As well, the stained gel was quantified by densitometry to determine the total amount of LHCII present in each lane. The peak heights were measured, background subtracted and the relative amount of radiolabel incorporated was calculated by dividing the value obtained from the autoradiogram by that from the stained gel. Thus, the content of radiolabel incorporated into LHCII was calculated as a "pseudo"-specific activity, based on the amount of stained LHCII in the gel lane. This compensated for any gel loading artifacts. Finally, the data were normalized from zero to unity within each experiment to allow averaging of the experimental repeats.

2.6. Separation of stromal and granal thylakoid membranes

Stromal and granal thylakoids were isolated as described by Berthold et al. [41] and adapted by Braun et al. [42] for Spirodela. Whole plants were placed on medium containing [^{32}P]orthophosphate (50 μCi/ml), incubated in 720 nm light for 1 h and subsequently placed in 640 nm (PS II) light for time periods up to 20 min. Immediately after harvesting a plant sample at a given time point, the tissue was homogenized at 4°C in 500 μl of a buffer consisting of 0.4 M sucrose, 10 mM Tricine (pH 8.0), 10 mM NaCl, 10 mM $MgCl_2$, and 10 mM NaF with a glass/glass tissue grinder. All remaining steps were carried out at 4°C. The homogenate was centrifuged for 10 s at 12000 × *g* in a microcentrifuge to pellet unbroken cells and debris. The supernatant was centrifuged at 12000 × *g* for 15 min to obtain the thylakoid fraction. The membrane fraction was washed with 500 μl of 20 mM Tricine (pH 8.0), 150 mM NaCl, 10 mM $MgCl_2$ and centrifuged at 12000 × *g*. The resulting pellet was suspended in 20 mM Mes (pH 6.5), 10 mM $MgCl_2$ to a final chlorophyll concentration of 2 mg ml^{-1}. Chlorophyll was assayed in 80% acetone according to Lichtenthaler [43]. Triton X-100 was added from a 30% (w/v) stock solution to give a chlorophyll:Triton X-100 ratio of 1:25 (w/w), the suspension was mixed gently and then incubated for 20 min in the dark without further agitation. This suspension was centrifuged for 30 min at 12000 × *g*. The resulting supernatant contained the stromal lamellae and the pellet contained the stacked (or granal) membranes. We have found that with Spirodela membranes the separation of granal and stromal membranes is efficient. There are minimal amounts of granal margins in the stromal membrane fraction, as shown by the exceedingly low levels of Coomassie blue stainable LHCII in the stromal fractions (see below). It has also been suggested that the Triton X-100 fractionation method can be optimized to limit the amount of granal margins solubilized [44].

3. Results

3.1. State transitions in vivo in Spirodela oligorrhiza as monitored by 77 K fluorescence

To determine whether Spirodela carries out state transitions and whether the process could be detected with intact plants using 77 K fluorescence, plants were incubated for 1 h either in 640 nm red light (PS II light) or 720 nm far-red light (PS I light). The plants were frozen at 77 K and fluorescence emission spectra were acquired from 650 nm to 800 nm (Fig. 1). Fluorescence emission peaks were detected at 685 (F685) and 695 (F695), representing Chl *a* associated with PS II, and at 735 nm (F735) representing Chl *a* associated with PS I [1,38]. The F685 peaks in the state 1 and state 2 samples were normalized, revealing that in state 2 there was a relative increase in fluorescence yield

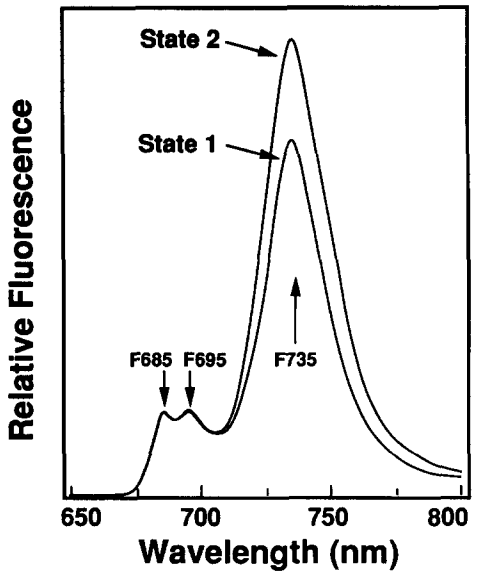

Fig. 1. Measurement of state 1 and state 2 in *Spirodela oligorrhiza* by low temperature (77 K) fluorescence spectroscopy. Plants were incubated in PS II (640 nm) or PS I (720 nm) light for 1 h and frozen at 77 K. Fluorescence was measured with an excitation wavelength of 435 nm and emission spectra were collected from 650 nm to 800 nm. Three characteristic peaks were observed: F685, F695 and F735. The spectra were normalized to F685 to show the difference between the states more clearly at F735. The extent that the plants are in state 1 or 2 can be quantified as the ratio between the F735 and F685 peaks. In state 2, the F735/F685 ratio is approx. 5 and in state 1 the ratio is about 4.

from PS I (F735) and/or a decrease in yield from PS II. In state 2 the F735/F685 ratio was high (approx. 5) and in state 1 the ratio was low (approx. 4). This change in relative Chl *a* fluorescence emission from PS II and PS I is typical of in vitro state transitions [9,10,13,14,16]. The values of F735/F685 we observed are high due to selective self-absorption of 685 nm fluorescence by chlorophyll in the leaf. The F735/F685 ratio is thus dependent on the chlorophyll concentration of the plant tissue as well as on state transitions. We found Spirodela to be highly consistent in that the F735/F685 ratio was very similar among different batches of plants, that plants from the same batch culture had essentially identical F735/F685 ratios, and furthermore that the F735/F685 ratio only changed as a function of a state transition. Thus, the F735/F685 ratio was used in the remaining experiments to follow state transitions.

3.2. In vivo response spectrum for the state transition in Spirodela

In the above experiment, state 2 and state 1 were established with 640 nm and 720 nm light, respectively. It was of interest to determine which state was reached throughout the photosynthetically active range of the spectrum (400–740 nm) as this might indicate which

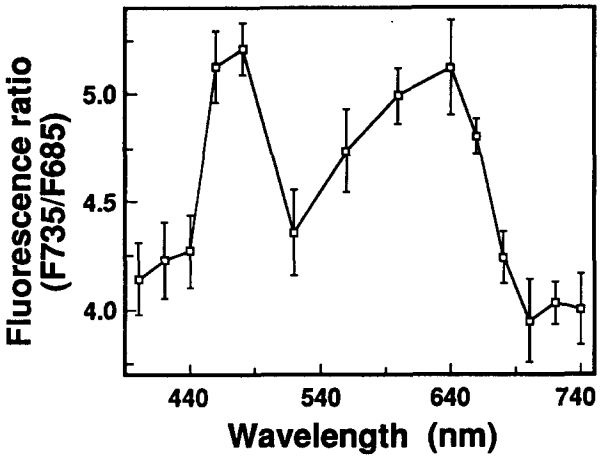

Fig. 2. Response spectrum for state transitions. The F735/F685 ratio was determined as a function of wavelength. Intact plants were placed in different wavelengths of photosynthetically active radiation for 1 h (all at 10 μmol m^{-2} s^{-1}) and then 77 K fluorescence emission spectra were measured. Each data point represents the mean of the F735/F685 ratio from three independent experiments and error bars are standard error of the mean ($n = 3$).

pigments trigger a state transition. Spirodela plants were transferred from visible light to the test wavelength, incubated for 1 h and analyzed by 77 K fluorescence spectroscopy. The resultant F735/F685 ratio was calculated for each wavelength. The response spectrum revealed two peaks for state 2; at 450–480 nm and at 600–640 nm (Fig. 2). This corresponds to the peak absorbance positions of Chl *b* [45], which is in higher

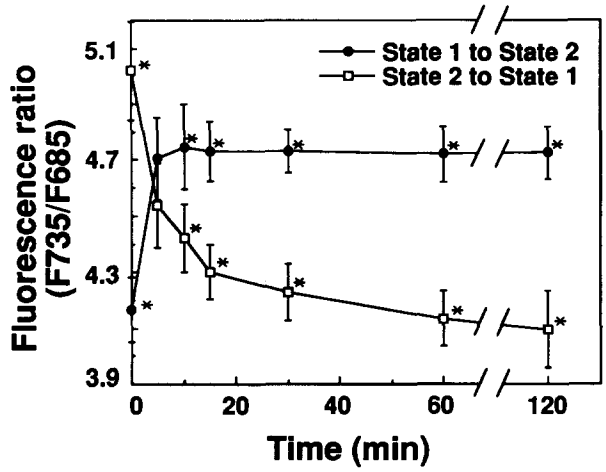

Fig. 3. Kinetics of state transitions in intact leaves of Spirodela as measured by 77 K fluorescence. Plants were incubated either in PS I light (720 nm) for 1 h and then transferred to PS II (640 nm) light (●), or in PS II light for 1 h and then transferred to PS I light (□). Plant samples were taken periodically over the subsequent 2 h period and analyzed by 77 K fluorescence. The data points are the ratio between the F735 and F685 emission signals, and represent the average of at least three independent experiments. Error bars are the standard error of the mean. Data points with an asterisk (*) represent means from the two treatments at identical time points that were significantly different from each other ($P \leq 0.05$).

proportions in PS II than in PS I [1]. In contrast, state 1 (minima in the F735/F685 ratio) was observed at 400–440 and 680–740 nm where Chl *a* absorbance was dominant. Absorbance by Chl *a* and Chl *b* is weak but nearly equal at 560 nm, and coincidentally an intermediate state between state 1 and state 2 was observed.

3.3. Kinetics of state transitions and LHCII phosphorylation

The data above showed a relationship between absorption by Chl *b* and achieving state 2, but did not address phosphorylation of LHCII. The kinetics of both the fluorescence and phosphorylation changes during in vivo state transitions were compared. Following incubation in PS I light (720 nm), the F735/F685 ratio was low (approx. 4), and upon transfer to PS II light (640 nm) the ratio increased rapidly (5 min) to a plateau of approx. 5 (Fig. 3). Conversely, plants initially incubated in 640 nm light (state 2) had a high F735/F685 ratio (approx. 5.0), and when these plants were transferred to 720 nm light the F735/F685 ratio dropped over 30 min until it stabilized at a ratio of about 4 (Fig. 3). Note, the $t_{1/2}$ for the state 1 to state 2 transition was significantly shorter than for the state 2 to state 1 transition (Table 1).

The kinetics of phosphorylation and dephosphorylation of LHCII were assayed under the same conditions as above. Plants were incubated in PS I or PS II light in the presence of [^{32}P]orthophosphate and subse-

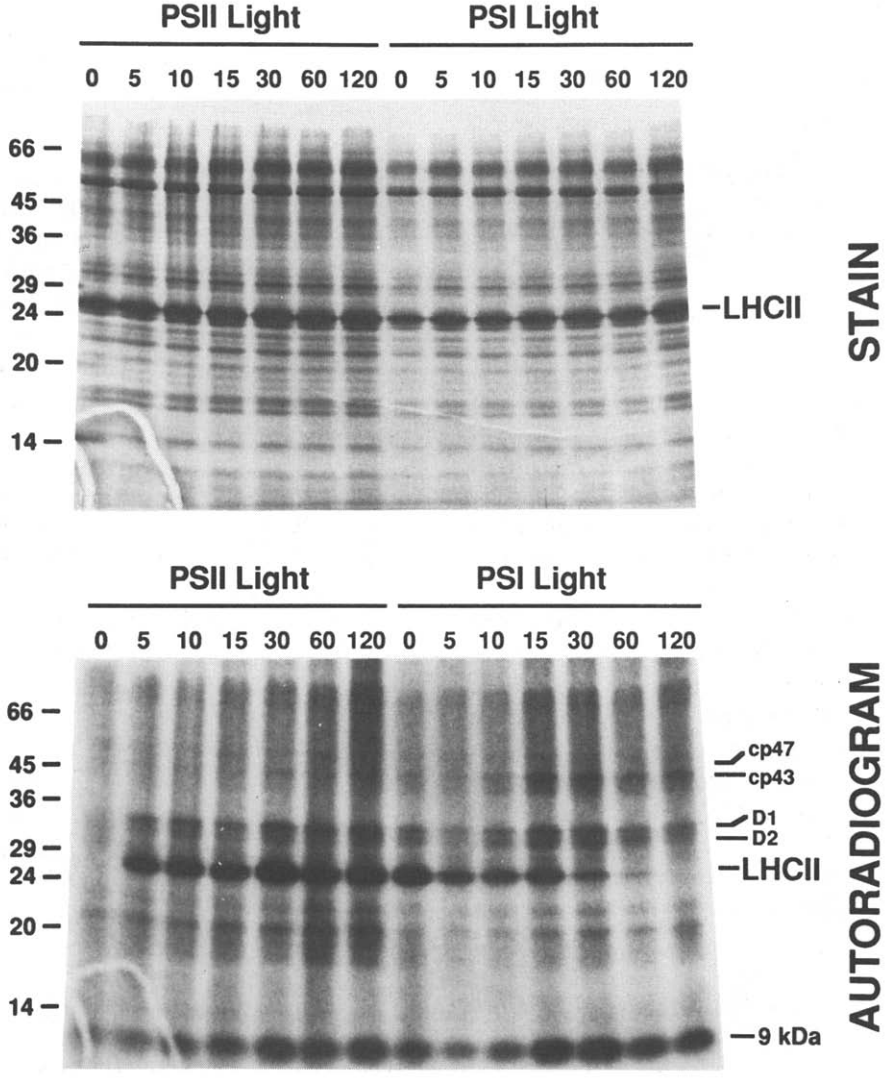

Fig. 4. In vivo LHCII phosphorylation and dephosphorylation during state transitions. Plants were incubated in either PS II (640 nm) or PS I (720 nm) light for 1 h in the presence of [^{32}P]orthophosphate. They were then placed in the opposite (chase) light condition (as indicated above the gels) on media without radiolabel and tissue was harvested periodically over a 2 h period. The numbers above each gel lane are the time points (min) at which the plants were harvested. Thylakoid membrane proteins were isolated, separated by SDS-PAGE, and detected by Coomassie blue staining (STAIN) and autoradiography (AUTORADIOGRAM). Positions of LHCII and molecular weight markers are indicated next to the gel. Positions of putative phosphorylated PS II core proteins (9 kDa phosphoprotein, D1, D2, cp43 and cp47) are marked.

quently moved to the chase light in the absence of radiolabel. With Spirodela, vacuum infiltration was not required for assimilation of the radiolabel, as is often the case with detached leaf segments and disks. Analysis of LHCII phosphorylation revealed that plants incubated with [^{32}P]orthophosphate in 720 nm light (PS I illumination) showed little label associated with LHCII (Figs. 4 and 5). However, upon transfer to 640 nm light (state 2 conditions), there was a rapid incorporation of radiolabel into LHCII. The half-time for the transition from the non-phosphorylated to the phosphorylated state was 4 min, similar to the rise time for the F735/F685 ratio (Table 1). For the state 2 to 1 transition, LHCII was maximally phosphorylated after incubation of the plants in PS II light. Upon transfer to PS I light the amount of radiolabel incorporated into LHCII gradually declined to background levels (Figs. 4 and 5). The half-time for dephosphorylation (13 min) was similar to the half-time for the transition to state 1 as measured by fluorescence (Table 1). Note, LHCII was the only phospho-protein apparent in the gels that was dephosphorylated within the time frame of a state transition. This included other PS II proteins, such as D1, D2, cp43 and the 9 kDa phosphoprotein, which are known to be phosphorylated in vitro and in vivo [19,20,22,46–48].

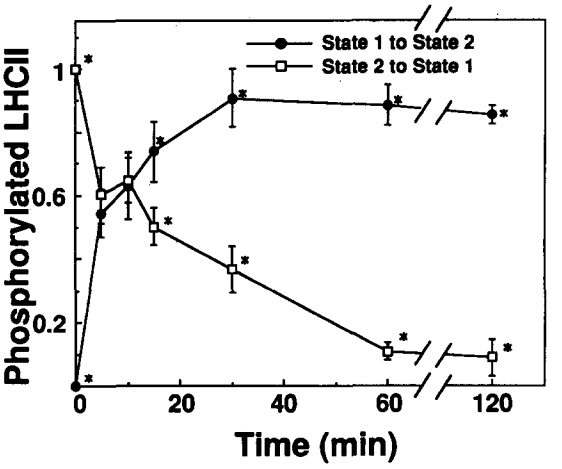

Fig. 5. Kinetics of LHCII phosphorylation and dephosphorylation. Plants were incubated in either PS I or PS II light for 1 h and then moved to the other light condition as described in Fig. 3. State 2 to state 1 transition (640 nm light to 720 nm light) (□). State 1 to state 2 transition (720 nm light to 640 nm light) (●). The Coomassie blue stained SDS-PAGE gels and corresponding autoradiograms from three experiments identical to that in Fig. 4 were scanned using a laser densitometer. The ratios of radiolabeled LHCII protein to Coomassie stained LHCII protein were determined. The ratios from individual experiments were normalized from 0 to 1 to allow averaging between experiments. Each data point represents the average of three independent experiments. Error bars represent standard error of the mean. Data points with an asterisk (*) represent means from the two treatments at identical time points that were significantly different from each other ($P \leq 0.05$).

3.4. Effect of NaF and DCMU on state transitions

To further address whether phosphorylation can be correlated with a state transition and the observed changes in chlorophyll fluorescence, inhibitors of both phosphorylation and dephosphorylation were employed. The phosphatase that dephosphorylates LHCII can be inhibited by NaF [12,15,19]. To investigate whether this phenomenon can be observed in vivo, the ability of NaF to inhibit a state 2 to 1 transition in Spirodela was determined by 77 K Chl *a* fluorescence and LHCII dephosphorylation (Fig. 6). In the absence of NaF, the F735/F685 ratio declined in 720 nm light to the state 1 level (Fig. 6A). However, in the presence of NaF, this ratio did not decline, indicating the plants stayed in state 2 under PS I illumination. When phosphorylation experiments were carried out under the same conditions, NaF blocked dephosphorylation of LHCII in PS I light (Fig. 6B). Thus, when the phosphatase was blocked in vivo, there coincidentally was no redistribution of light energy back to PS II.

As a control, a state 1 to state 2 transition was carried out in the presence of NaF (Fig. 7). Under these conditions, the kinase responsible for LHCII phosphorylation should function and a state 1 to 2 transition should occur. Indeed, when plants were switched from 720 nm light to 640 nm light in the presence of NaF the F735/F685 ratio increased, showing that the state 1 to state 2 transition could operate under these conditions. Interestingly, the F735/F685 ratio increased beyond plateau state 2 level observed in the control (Fig. 7A), implying that in the absence of an exogenous inhibitor the phosphatase maintains activity under state 2 conditions. The corresponding phosphorylation experiment revealed that phosphorylation of LHCII was not blocked by NaF and in fact the protein may have been phosphorylated to a greater extent than in the control plants (Fig. 7B). This indicates that the kinase operates in the presence of NaF.

Table 1
Half-times for state transitions as measured by 77 K fluorescence or LHCII phosphorylation

Technique	State transition	$t_{1/2}$ (min) [1]	ANOVA grouping [2]
77 K fluorescence	2 to 1	10±1.1	a
	1 to 2	3±0.5	b
LHCII phosphorylation	2 to 1	13±1.7	a
	1 to 2	4±0.6	b

[1] $t_{1/2}$ represents the means of half-times calculated individually from each state transition experiment ± S.E. For fluorescence $n = 6$ and for phosphorylation $n = 3$.

[2] Means with the same letter indicate that a two-way analysis of variance was not significant at the 95% confidence level. Means with different letters are significantly different at the 95% confidence level.

Direct inhibitors of the LHCII kinase that work in vivo have not been characterized. However, DCMU can be used indirectly to inhibit phosphorylation since it inhibits electron flow through PS II and will lead to oxidation of the PQ pool under PS II light [24,33,36,49]. Under these conditions the cytochrome b_6/f complex also will be in a net oxidized state. This is known to inactivate the kinase [14,21], which should trigger a transition from state 2 to state 1 under PS II illumination. To test whether this occurred in vivo, the tissue was incubated in 640 nm light for 1 h and DCMU was added to the media. The plants were left in PS II light and tissue was harvested periodically for 77 K fluorescence and LHCII phosphorylation analyses. The plants incubated in PS II light had a high F735/F685 ratio of about 5 (Fig. 8A). However, following the addition of DCMU, the ratio gradually decreased to a state 1 level. Thus, the addition of DCMU triggered a state 2 to state 1 transition in PS II light. The phosphorylation

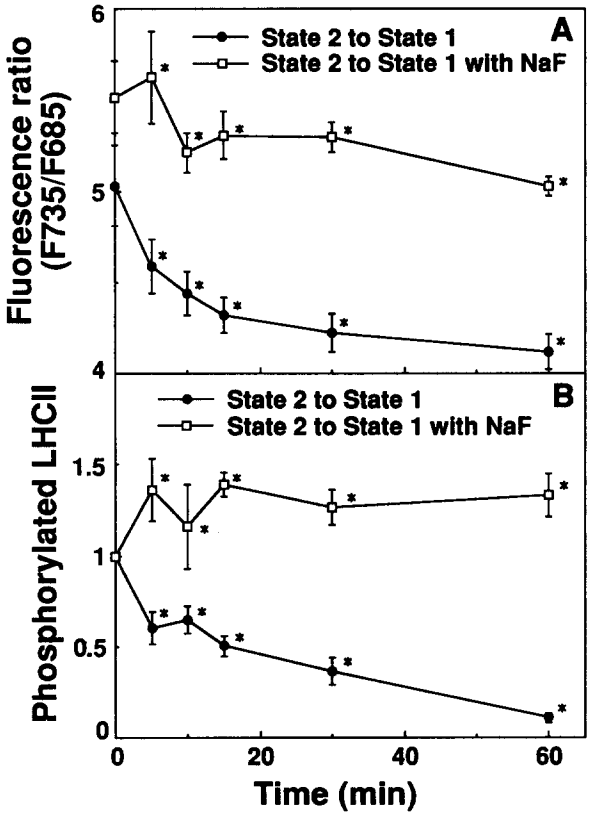

Fig. 6. The effect of NaF on the transition from state 2 to state 1 in vivo. Plants were brought to state 2 by incubation in 640 nm light for 1 h and then placed in media with (□) or without (●) 125 mM NaF and incubated in PS I light. Plant samples were taken at various time points for either F735/F685 ratio determinations (A) or quantification of LHCII dephosphorylation (B). Each data point is the average of at least three independent experiments. Error bars represent standard error of the mean. Data points with an asterisk (*) represent means from the two treatments at identical time points that were significantly different from each other ($P \leq 0.05$).

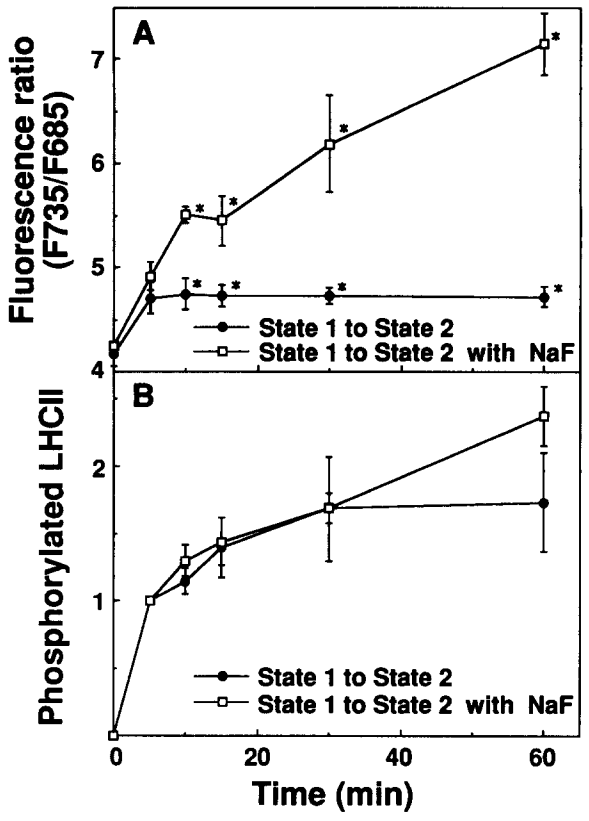

Fig. 7. The effect of NaF on the transition from state 1 to state 2 in vivo. Plants were brought to state 1 by incubation in 720 nm light for 1 h and then placed in PS II light without NaF (●) or in PS II light with 125 mM NaF (□). Plant samples were taken at various time points and the F735/F685 ratio was determined by 77 K fluorescence (A) or LHCII phosphorylation was monitored (B). Each data point is the average of at least three independent experiments. Error bars represent standard error of the mean. Data points with an asterisk (*) represent means from the two treatments at identical time points that were significantly different from each other ($P \leq 0.05$).

experiments supported this observation (Fig. 8B), as the level of LHCII phosphorylation declined in plants incubated in PS II light with DCMU present.

3.5. LHCII migration during a state transition in vivo

It has been postulated that LHCII phosphorylation leads to migration of the phosphorylated protein from stacked grana where PS II is predominantly located, to unstacked stromal thylakoids where PS I dominates [5,6,9,12,14,15]. The phosphorylated LHCII would then associate with PS I, increasing the absorption cross-section of PS I. This theory was first tested by measuring the absolute (as opposed to relative) levels of the 77 K fluorescence emission signals from PS II and PS I in state 1 and state 2. Mechanical sampling differences were minimized (see Materials and methods), so that the only source of variation in the experiment was the

leaf tissue. Plants were incubated in either PS I or PS II light for 1 h and eight individual plant samples from each treatment were analyzed. The resultant scans from plants in each state were averaged and plotted without normalization (Fig. 9). The signal emitted by PS I (735 nm) was 11.7% higher in state 2 than in state 1 (this difference was significant, $P = 0.012$). Consistent with the higher signal from PS I in state 2, the signal from PS II was 12.7% lower in state 2 than in state 1 (this difference was also significant, $P = 0.029$). Therefore, in state 2 versus state 1, there was an absolute increase in fluorescence from PS I along with an equal and opposite change in fluorescence from PS II. The data indicate an alteration in cross-sectional areas of the antenna on both photosystems, and imply that in vivo the LHCII complex was indeed migrating between the two photosystems or there was an efficient spillover mechanism in place.

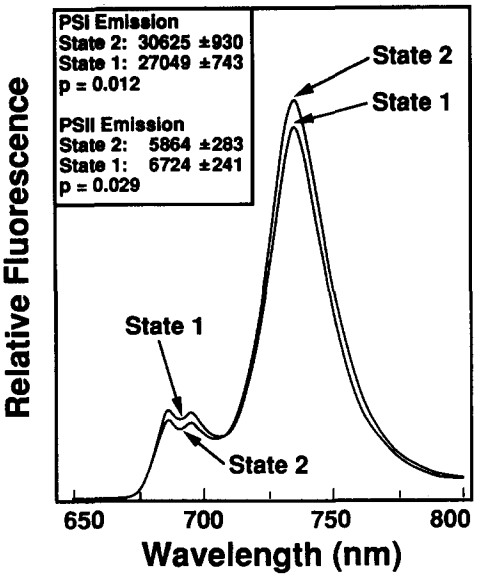

Fig. 9. State 1 and state 2 measured in intact leaves of Spirodela by 77 K fluorescence without normalization. Plants were incubated in PS II (640 nm) light or PS I (720 nm) light for 1 h. Low temperature fluorescence emission spectra of eight plants from each light treatment were collected, averaged and plotted without further normalization. Inset: averaged absolute signals from the diode array detector for PS I (F735) and PS II (F685). The resulting averaged scans were significantly different ($P < 0.05$).

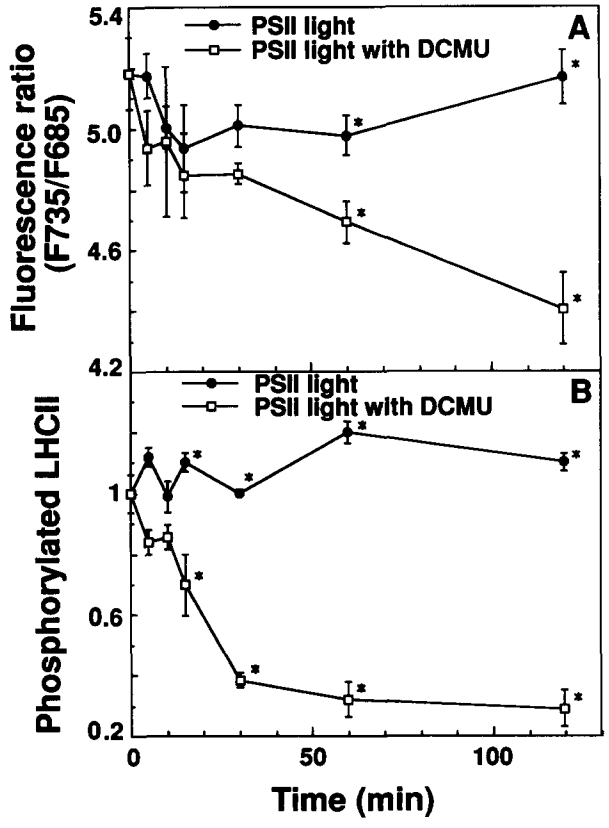

Fig. 8. Induction of state 1 by DCMU in PS II light. Plants were brought to state 2 by incubating in 640 nm light for 1 h and then maintained in PS II light either with (□) or without (●) 50 μM DCMU. Plant samples were taken at various time points after the addition of DCMU. The F735/F685 ratio was determined by 77 K fluorescence (A) or LHCII dephosphorylation was monitored (B). Each data point is the average of at least three independent experiments. Error bars represent standard error of the mean. Data points with an asterisk (*) represent means from the two treatments at identical time points that were significantly different from each other ($P \leq 0.05$).

Although not a proof of functional association with PS I, if phospho-LHCII was actually migrating to PS I, then some of the phosphorylated LHCII should be found in the unstacked membranes. Plants were incubated in PS I light for 1 h with [^{32}P]orthophosphate and transferred to PS II light. Tissue was collected at various time points, and the thylakoid membranes were separated into the unstacked and stacked fractions (Fig. 10). The unstacked membranes appeared to be essentially free of granal thylakoids and probably granal margins, as exceedingly small amounts of LHCII were detectable in the stromal thylakoids by Coomassie blue staining (Fig. 10A). The pattern of stainable proteins in the two types of membrane fractions did not change during a state transition, so only one example of each membrane fraction is shown in the Coomassie stained gel. LHCII phosphorylation in stacked thylakoid membranes was readily detected within 1 min in 640 nm light and increased with time (Fig. 10B). However, in the unstacked membranes phosphorylated LHCII was not detected until 3 min and saturated at about 10 min, roughly equivalent to the kinetics of a state 1 to state 2 transition. Note that the autoradiograms of the gels of both membrane fractions are shown at approximately equal exposures (similar background levels throughout and similar signals of LHCII at saturation), so if radiolabeled LHCII was present even at a modest level in the stromal membrane fraction at 1 min it would have

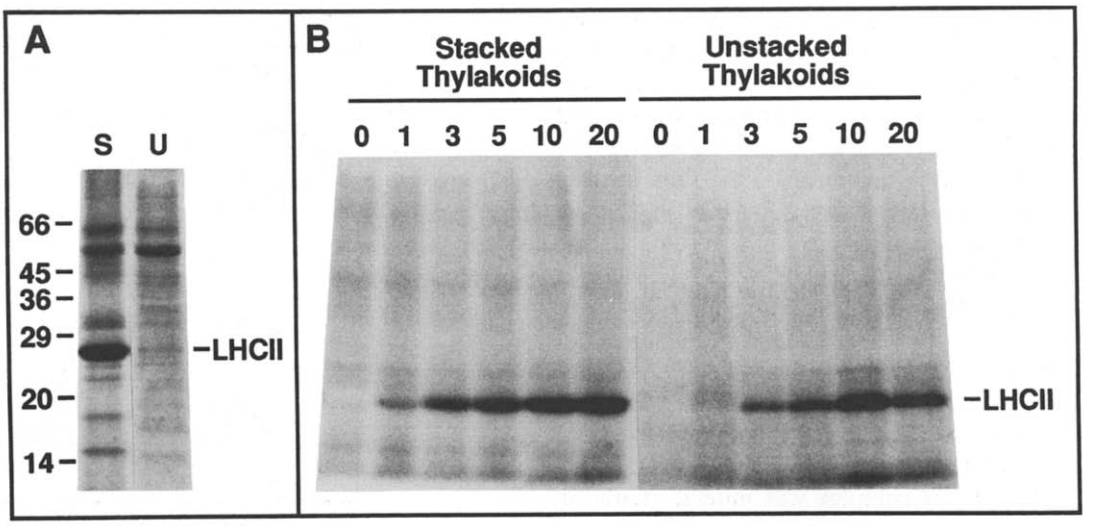

Fig. 10. Phosphorylation of LHCII in stacked and unstacked thylakoids during a state 1 to state 2 transition. Plants were incubated in PS I light for 1 h in media containing [^{32}P]orthophosphate. The plants were placed on fresh media without radiolabel and incubated in PS II light. Tissue was collected and homogenized immediately for membrane fractionation. Numbers above the gel lanes indicate the time points (min) at which the tissue was harvested. The membranes were isolated, and separated into the stacked (granal) thylakoids and unstacked (stromal) thylakoids. The proteins were analyzed by SDS-PAGE. (A) Coomassie blue stained gel of representative samples of stacked (S) and unstacked (U) thylakoids. (B) Autoradiographic detection of phosphorylation of LHCII in stacked and unstacked thylakoids during a state 1 to 2 transition. Positions of LHCII and molecular weight markers are indicated.

been detected. We thus conclude that there is a lag in appearance of phospho-LHCII in stroma exposed membranes, which is consistent with LHCII migration from granal thylakoids. Note, however, that not all the phospho-LHCII was observed to translocate to the unstacked membranes.

4. Discussion

Spirodela oligorrhiza has the ability to redistribute photosynthetic light energy in vivo through state transitions. A change in the fraction of photons absorbed by PS I is reflected as an increase in low temperature PS I-associated Chl *a* emission (F735) relative to PS II-associated Chl *a* emission (F685 and F695). This process appears to be regulated by the differential absorption of Chl *a* and Chl *b*, with the signal transduced via the phosphorylation state of LHCII. Upon phosphorylation, the LHCII protein would migrate from the stacked to the unstacked thylakoids. Thus, one of the models involving phospho-LHCII migration and association with PS I that has been observed in vitro and in leaf disks [5–7,10,12,24] operates in vivo in whole plants.

The response spectrum for state transitions (see Fig. 2) reveals that light predominantly absorbed by Chl *b* (450–480 and 640 nm) results in state 2. PS II, due to its abundance of LHCII, contains much more Chl *b* than PS I [1]. When Chl *b* excitation is favored, PS II receives more light than PS I, net reduction of the cytochrome b_6/f complexes occurs and the LHCII kinase is activated [21]. Conversely, the minima in the state 2 response spectrum (400–440 nm and 700–740 nm) show that light absorbed preferentially by Chl *a*, which is in higher proportions in PS I, leads to state 1. This is because the PQ pool will be in a net oxidized state when PS I is preferentially excited. Finally, when the absorbance of the two photosystems is nearly equal (560 nm), the PQ pool is in an intermediate oxidation state, and an intermediate between state 1 and 2 is observed. Thus, transition to state 1 or 2 in vivo is dictated by which photosystem is preferentially illuminated.

Our results show that the LHCII phosphorylation/dephosphorylation phenomenon that has long been connected to state transitions in vitro and with leaf disks [5,6,10,12,14–16,19], also occurs in vivo in whole plants. In particular, the kinetics of state transitions, as measured by fluorescence in whole Spirodela plants correlated well with changes occurring in the phosphorylation state of LHCII (Table 1). LHCII is essentially the only thylakoid protein that shows dephosphorylation with kinetics coincident with a state 2 to 1 transition (see Fig. 4). For instance, proteins that run in the positions of the core proteins of PS II (D1, D2, cp43, cp47 and the 9 kDa PS II phospho-protein) remain phosphorylated in PS I light. It has been shown by others that D1, D2, cp43 and the 9 kDa PS II protein are phosphorylated in vivo [19,20,22,46–48]. Further, dephosphorylation of the D1 and 9 kDa proteins were reported to occur under PS I light, but with kinetics much slower than a state 2 to 1 transition [19,23,48]. Thus, while phosphorylation of other PS II proteins may be involved in regulation of PS II, their phospho-

rylation is unlikely to be connected to state transitions in vivo. To the best of our knowledge this is the first report that cp47 is phosphorylated. However, the band is only weakly radiolabeled and our conclusion is based strictly on relative mobility in SDS-PAGE.

To further establish the relationship between phosphorylation/dephosphorylation and state transitions, inhibitor studies of both the phosphatase and the kinase were undertaken. The state 2 to state 1 transition in vivo as measured by 77 K fluorescence was shown to be inhibited by NaF. This inhibitor seems to block the phosphatase that catalyzes the dephosphorylation of LHCII (Fig. 6). It should be noted that if in vivo NaF blocked any process downstream from the PQ pool (e.g. the Calvin cycle), the result would be maintenance of a reduced PQ pool and thus the LHCII phosphatase would remain inactive (or the kinase would remain activated in PS I light). This was found to be the case for D,L-glyceraldehyde which inhibits the Calvin cycle and keeps LHCII phosphorylated in PS I light [49]. Nonetheless, whether NaF blocks dephosphorylation directly or indirectly, simultaneous inhibition of a state 2 to 1 transition and dephosphorylation was observed. DCMU does not allow reduction of the PQ pool [36] and prevents activation of the LHCII kinase [24,49]. Intact plants incubated under PS II light were found to achieve state 1 simply by the addition of DCMU. Together the two inhibitor studies support the in vitro correlation between phosphorylation/dephosphorylation of LHCII and energy redistribution during state transitions. This is the first time this correlation has been demonstrated in an intact plant system.

The requirement for phosphorylation and dephosphorylation of LHCII during a state transition emphasizes the importance of regulation of the kinase and/or the phosphatase. Normally a transition to state 2 plateaus at a steady state level after about 5 min. However, in the presence of NaF the F735/F685 ratio continues to rise to a more extreme state 2 level (Fig. 7). This was also reflected by phosphorylation of LHCII, albeit to a lesser extent. The enhanced state 2 is perhaps not shown as effectively by the phosphorylation data because only a fraction of the phospho-LHCII moves to the stromal lamellae (see Fig. 10). It appears that the phosphatase is only fully inactivated under PS II light when an exogenous inhibitor is present. It has been reported that in vitro the LHCII phosphatase is less sensitive than the kinase to changes in the redox state of the PQ pool [9,19]. It is possible that the phosphatase is constitutively active, and that state transitions are mostly controlled at the level of the kinase. This contrasts with dephosphorylation of the D1 protein, which is suggested to be activated by PS I [48]. Thus, our in vivo data support previous in vitro experiments reporting relatively little regulation of the LHCII phosphatase by light.

Experiments on state transitions using in vitro systems, leaf disks or detached leaves suggested that the absorbance cross-sections of PS I and PS II change in a reciprocal manner during the transition [3,5,6,9,13,15, 18]. The in vivo Chl *a* fluorescence measurements presented here support this theory. When 77 K fluorescence spectra from plants in either state 1 or state 2 were averaged without normalization, significant differences in emission from both PS I and PS II were obtained (Fig. 9). State 2 results in an increased absolute fluorescence signal emanating from PS I at the expense of PS II fluorescence. Conversely, state 1 results in an increased signal from PS II at the expense of PS I fluorescence.

The lag in the appearance of phospho-LHCII in stromal thylakoids following its phosphorylation in the granal stacks (Fig. 10) strongly supports the hypothesis that LHCII migrates between the two membrane types. Because of the difficulty in isolating stromal thylakoids free of granal margins [44], we cannot rule out the possibility that LHCII is only migrating to granal margins. However, there is little stainable LHCII in the stromal fractions (see Fig. 10A), so we are confident that the content of granal margins is minimal. The complementary fluorescence data (see Fig. 9) indicate that when phospho-LHCII reaches the stromal exposed membranes it associates with PS I. However, the mechanism of LHCII migration following a change in its phosphorylation state remains unclear. It has been proposed that when phosphorylated, LHCII dissociates from PS II and moves freely from the stacked to unstacked membranes where it binds to PS I [9,10,14, 15,25]. However, to the best of our knowledge there are no reports of transient increases in either fluorescence or heat emission from lone LHCII molecules during state transitions. Thus, it is possible that LHCII is associated with a photosystem during migration. Perhaps phosphorylation of LHCII triggers dissociation from PS II α centers and reassociation with PS II β centers. These complexes could carry LHCII to the stromal thylakoids [33,34], at which point there would be association to PS I. Another possibility is that the rate limiting step of a state transition is PS II-LHCII dissociation, with migration being a fairly rapid step. Nonetheless, it appears that in vivo LHCII physically migrates between the two photosystems during a state transition.

Our work makes the association between LHCII phosphorylation/migration and state transitions stronger by connecting in vitro evidence to in vivo whole plant measurements. The in vivo mechanism derived here is based on kinetic analyses, use of inhibitors and fractionation of chloroplast components, connecting fluorescence and LHCII phosphorylation in several parallel experiments. In particular, LHCII is the only thylakoid protein whose in vivo dephosphory-

lation can be correlated to state transitions. It is interesting to consider that phosphorylation has been suggested as a potential signal for degradation of the D1 PS II reaction center protein under non-saturating fluence rates in vivo [50], with removal of the phosphate immediately preceding D1 protein degradation under photoinhibitory fluence rates in vitro [51]. Thus, phosphorylation could be a primary in vivo mechanism for selective protein trafficking and cycling in thylakoids.

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