Chapter 17

Probing the Mechanism of State Transitions in Oxygenic Photosynthesis by Chlorophyll Fluorescence Spectroscopy, Kinetics and Imaging

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Summary

The machinery of oxygenic photosynthesis can move between two light-states. State 1 is induced by a light regime favoring Photosystem (PS) I, and favors light-harvesting by PS II. State 2 is induced by a light regime favoring PS II, and favors light-harvesting by PS I. Chl fluorescence emission is an important and revealing signature of the modifications that occur to photosynthetic unit structure and function during transitions between

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these two states. State 1 and state 2 are physiological adaptations to wavelength and to metabolic demands placed upon photosynthesis: they involve post-translational modification of pre-existing proteins, and restructuring of photosynthetic units. Chl fluorescence can be used to probe the signals initiating state transitions, their mechanisms, and the components of the redox signal transduction pathways involved.

I. Introduction to State Transitions

A. Physiological Role

PS I and PS II are connected in series, and so, electrons must flow through them at equal rates. In all green plants and most eukaryotic algae, PS I uses blue, red and far-red light, while PS II uses more blue and red light, but almost no far-red light. In cyanobacteria and red algae, most (around 85%) of the Chl is associated specifically with PS I, while the major part of the antenna of PS II usually consists of the linear tetrapyrrole pigments, phycobilins. In contrast to Chl, phycobilins absorb green and yellow-orange light, which is therefore selective for PS II. Where phycobilins are present, light absorbed by Chl is selective for PS I. In all systems, therefore, the two photosystems differ in their absorption and action spectra, and, experimentally, it is possible to define spectral bands selective for one or the other photosystem. In natural environments, the intensity and spectral composition of ambient light fluctuate with time, notably because of changes in shading and, for aquatic environments, in spectral filtering by water. If some of the energy available is not to be wasted when one photosystem becomes rate-limiting to the other, then there must be some way of redistributing light-harvesting antenna molecules to achieve balanced distribution of energy between the photosystems For an introduction to photosynthesis, see Blankenship (2002)

Short-term physiological adaptation of this kind can be induced experimentally when plants, algae or cyanobacteria are subjected to altered illumination conditions. The mechanism of this adaptation involves redistribution of absorbed excitation energy between the two photosystems, such that the light-limited photosystem receives more energy while the light-saturated photosystem receives less.

The first explicit description of physiological redistribution of absorbed excitation energy between PS I and PS II was obtained independently for the red alga *Porphyridium cruentum* (Murata, 1969) and the green alga *Chlorella pyrenoidosa* (Bonaventura and Myers, 1969). It is interesting to record that the same fundamental process appeared to operate irrespective of the type of 'accessory' pigment involved in light-harvesting (Chls *a* and *b* in *Chorella* and phycobilins in *Porphyridium*). Subsequent research suggests that 'state 1-state 2 transitions' are a universal property of organisms that live by means of oxygen-evolving photosynthesis, from cyanobacteria to higher plants (Williams and Allen, 1987; Allen, 1992).

The basic terminology associated with the phenomenon of state transitions stands independently of their mechanism (Myers, 1971). In all cases, PS I may be selected by a PS I-specific light, which can be termed 'light 1;' PS II is correspondingly selected by 'light 2.' The state of adaptation to light 1 is called the 'light 1-state' or 'state 1.' The state of adaptation to light 2 is called the 'light 2-state' or 'state 2.' The transition from state 2 to state 1 is called the 'state 1 transition.' By definition, the state 1 transition involves redirection of absorbed excitation energy to PS II, at the expense of PS I. The transition from state 1 to state 2 is called the 'state 2 transition,' and it involves redirection of absorbed excitation energy to PS I, at the expense of PS II.

B. Effects on Excitation Energy Transfer

Figure 1, adapted from the results of Bonaventura and Myers (1969), illustrates the process. Dark-adapted cells are illuminated with modulated light 2 at 645 nm. Chlorophyll (Chl) *a* fluorescence falls slowly from an initial maximum, and oxygen yield increases with approximately the same kinetics. This indicates a redistribution of excitation energy in favor of PS I , which is initially rate limiting but which becomes more effective in capturing light energy as

Abbreviations: Chl – chlorophyll; DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU – 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea; FRAP – Fluorescence Recovery After Photobleaching; LHCII – light harvesting Chl *a*/Chl *b* protein complex of Photosystem II; P680 – reaction center of Photosystem II; P700 – reaction center of Photosystem I; PQ – plastoquinone; PS – photosystem; Q_B – secondary plastoquinone electron acceptor of PS II

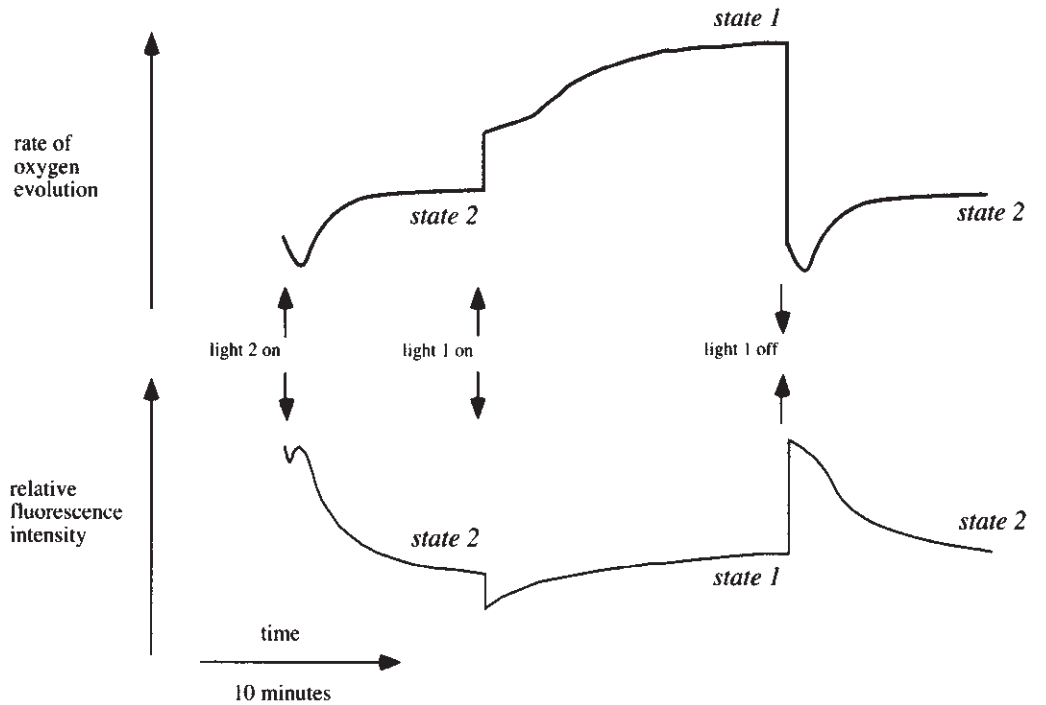

Fig. 1. Model state 1-state 2 transitions, schematically representing data of Bonaventura and Myers (1969) with *Chlorella pyrenoidosa*. The intensity of Light 2 (e.g., $\lambda = 645$ nm) is modulated and the synchronous oxygen and fluorescence signals are obtained through a lock-in amplifier. Effects of continuous light 1 (e.g., $\lambda = 710$ nm) are therefore indirect, and indicate redistribution of excitation energy between PS I and PS II. For fluorescence, qualitatively similar results can be obtained with any oxygen-evolving, two-light-reaction species, from cyanobacteria and cryptomonads to leaves of higher plants. The phenomenon can be demonstrated in isolated chloroplasts, and in isolated thylakoids provided ATP is present (at e.g., 200 μM) as a substrate for the LHC II kinase. The wavelengths described above are suitable for LHC II-containing organisms. In phycobilin-containing organisms light 2 should be specific for phycobilin absorption (e.g., within the range 500–610 nm) and any Chl-absorbed light (e.g., blue, between on 440 or 480 nm, or red, above 640 nm) will function as light 1. A terminal electron acceptor must be available (e.g., $NADP^+$ regenerated by assimilatory reactions in intact systems) if light absorbed by the PS I antenna is to function as light 1, and the light intensity should not be saturating. At saturating intensities or in the absence of PS I electron acceptors, any kind of light functions as light 2 by reducing plastoquinone.

the redistribution proceeds. Chl fluorescence from PS II decreases as a result of the combined decrease in excitation energy transfer to PS II and increased photochemical quenching of PS II fluorescence. The state of maximum oxygen yield under light 2 is state 2. The transition to state 2 is thus a process of redistribution of excitation energy in favor of PS I. Upon addition of continuous light 1 at 710 nm, further quenching of PS II fluorescence occurs. There is then a slow increase in oxygen yield accompanied by a fluorescence rise that indicates redistribution of excitation energy back to PS II. The new state of maximum oxygen yield under light 1 is state 1. The transition to state 1 is thus a process of redistribution of excitation energy in favor of PS II. In whole cells it is apparent that both the state 1 and state 2 transitions result in increased yield of oxygen (Fig. 1). Fluorescence measurements alone do directly register whether energy not released as fluorescence is lost or used, productively, for photochemistry. Similar fluorescence transients have now been recorded with a wide variety of cyanobacteria (Mullineaux et al., 1986), plants and algae.

In chloroplasts, the transition to state 2 can be explained by redox-controlled phosphorylation of a mobile component of light harvesting Chl *a*/Chl *b* protein complex of Photosystem II (LHC II), as follows. Where light 2 tend to drive PS II faster than PS I, plastoquinone becomes reduced, the LHC II kinase is activated, LHC II becomes phosphorylated, and phospho-LHC II migrates from PS II to PS I. Conversely, the transition to state 1 occurs because light 1 drives PS I momentarily faster than PS II, plastoquinone becomes oxidized, the LHC II kinase is inactivated,

and the LHC II phosphatase dephosphorylates LHC II thereby returning it to PS II (Allen, 1981).

The model of plastoquinone redox control of LHC II distribution during state transitions was prompted by the discovery of plastoquinone redox control of phosphorylation of LHC II (Allen et al., 1981). Early direct evidence in favor of this model was provided by Telfer et al. (1983) who used modulated fluorescence to follow state 1-state 2 transitions in pea thylakoids with ATP present at 0.15 mM to provide a substrate for protein phosphorylation. The fluorescence rise indicating the state 1 transition was inhibited by the protein phosphatase inhibitor NaF. Furthermore, state 2 was shown to be a state of high LHC II phosphorylation and state 1 a state of low LHC II phosphorylation, with the kinetics of changes in LHC II phosphorylation matching exactly the kinetics of the fluorescence changes, with a half-time of 4 min for the state 2 transition and 6 min for the state 1 transition.

C. Reorganization of Protein Complexes

Figure 2 shows a general scheme for control of excitation energy distribution by protein phosphorylation in phycobilisome-containing organisms (left). Any protein kinase catalyzing the protein phosphorylation(s) involved in the state 2 transition in cyanobacteria (a) is assumed to be under redox control, analogous to the LHC II kinase. Fig 2(b) shows the corresponding lateral re-arrangement of LHC II between Photosystems I and II of chloroplast thylakoids.

The functional effect of phosphorylation of LHC II first identified was a change in Chl fluorescence emission properties of isolated thylakoids (Bennett et al., 1980). Phosphorylation is accompanied by a decrease in total Chl fluorescence yield at room temperature which is consistent with decreased emission from

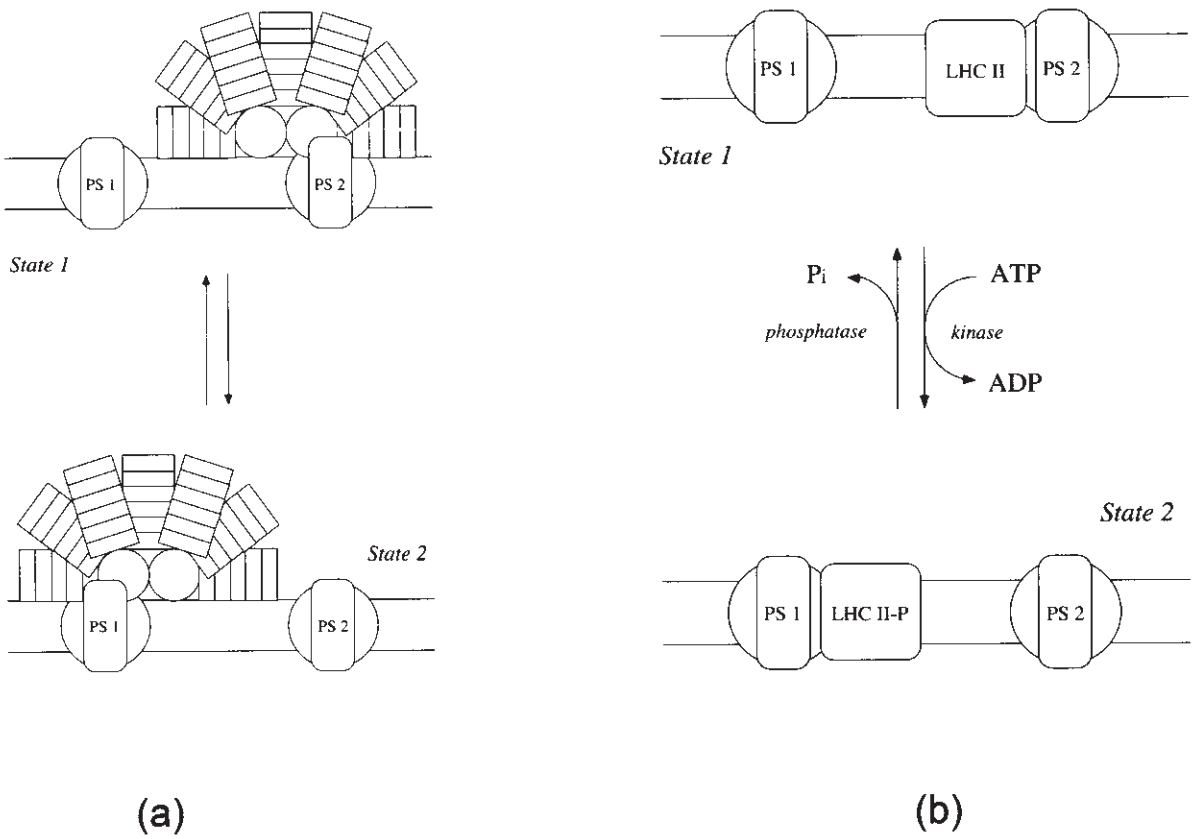

Fig. 2. Scheme for control of excitation energy distribution in phycobilisome-containing organisms (a) and in LHC II-containing organisms (b). The kinase catalyzing the LHC II protein phosphorylation(s) involved in the state 2 transition in (b) is under redox control. The chemical nature of the modification in phycobilisome-containing organisms (a) is not known, though it is likely that phosphorylation reactions are also involved.

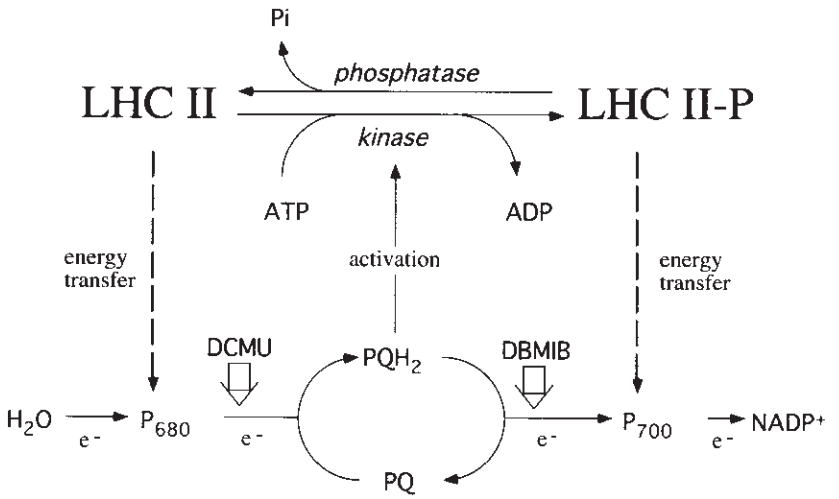

Fig. 3. Control of distribution of LHC II between Photosystems I and II by redox-activation of its protein kinase. Adapted from Allen (1992)

PS II. Fluorescence spectroscopy at 77 K shows that LHC II phosphorylation produces a decrease in yield in the PS II emission bands at 685 and 695 nm relative to that of the PS I band at 735 nm. Excitation energy from light absorbed by LHC II becomes diverted away from PS II as a result of phosphorylation, and all or part of that energy reaches PS I instead.

Normalization procedures initially showed only a relative increase in the ratio F_{735}/F_{685}. Using fluorescein as an external standard in isolated chloroplast thylakoids reveals that the LHC II phosphorylation underlying state transitions produces both a decrease in F_{685} and an increase in F_{735}. The same conclusion is supported using phycoerythrin as an external standard with *Chlorella* cells and thylakoids (Saito et al., 1983).

The structural basis of the movement of LHC II between Photosystems I and II in chloroplasts has been the subject of much debate.. A recent review stresses the role of steric effects and guided molecular recognition in determining the functional alignment of intrinsic membrane proteins (Allen and Forsberg, 2001). In cyanobacterial thylakoids, structural rearrangements have recently been visualized by a number of techniques, including FRAP (Fluorescence Recovery After Photobleaching), as described in Section IV.

D. Biochemical Mechanisms

Figure 3 shows control of distribution of excitation energy, absorbed by LHC II, between the reaction centers of PS I (P700) and PS II (P680). An LHC II kinase is activated when plastoquinone (PQ) is in its reduced form (PQH_2), resulting in phosphorylation of LHC II. The phosphorylated form of LHC II transfers excitation energy to PS I at the expense of PS II, serving to oxidize the plastoquinone pool. The LHC II phosphatase is redox-independent (Silverstein et al., 1993). When plastoquinone is oxidized, the kinase is inactive, and dephosphorylation of LHC II predominates, thus returning excitation energy to PS II and increasing the rate of reduction of plastoquinone. The plastoquinone pool is oxidized by PS I and reduced by PS II. Electron transport from PS II to plastoquinone is inhibited by 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU) that inhibits the reaction by displacing Q_B, the secondary plastoquinone electron acceptor of PS II; electron transport from plastoquinone to PS I is inhibited by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) that binds to a site on Cyt *b/f* complex. Adapted from Allen et al. (1981) and Allen (1992).

II. Studying State Transitions using Continuous Measurements of Fluorescence

Apart from fluorescence emission spectroscopy (Section IB), induction of continuous fluorescence on a millisecond time scale helps to delineate possible mechanisms of state transitions. In state 1, an extended excitation energy transfer pathway between PS II units is consistent with an observed sigmoidicity

in fluorescence induction kinetics. The transition to state 2 involves not only an increase in absorption cross-section of PS I and a decrease in that of PS II, but it also produces a decrease in the co-operativity among PS II units. This decreased co-operativity causes a decrease in sigmoidicity by increasing the contribution of a first-order, single-exponential rise to the kinetics of fluorescence induction. This change in co-operativity of photosynthetic units may even take place independently of complementary changes in absorption cross-section, and be a property of a related regulatory process in the single-photosystem purple bacteria (Holmes and Allen, 1988).

Figure 4 shows model fluorescence induction transients, illustrating the decrease in both F_m and F_o in state 2, such as upon phosphorylation of LHC II, and in F_m alone on cation depletion ($-Mg^{2+}$). The upper transients suggest a decrease in the absorption cross-section of PS II, the lower transients an increase in spillover from PS II to PS I (see section IIIB).

III. Studying State Transitions using Picosecond Fluorescence Kinetics

A. Introduction

State transitions modify the function of the light-harvesting complexes, thus changing the pathways of energy flow. In principle, picosecond time-resolved fluorescence measurements provide one of the best ways to obtain detailed information on photosynthetic energy transfer processes . The principle of the method is that the sample is excited by a very short laser excitation pulse, and fluorescence detected over picoseconds to nanoseconds after the pulse. The kinetics of fluorescence decay at different wavelengths should reveal the pathways of excitation energy migration. In practice, fluorescence decay from intact systems tends to be very complex, and careful data analysis is necessary. A relatively simple but effective method is to use 'global data analysis' (Wendler and Holzwarth, 1987). This involves measuring fluorescence decay at a series of emission wavelengths. The set of decays is deconvoluted together, on the assumption that each component has the same lifetime throughout, and only its relative amplitude is wavelength-dependent. The results can be presented as 'decay-associated spectra', which show the wavelength-dependence of the amplitude of each lifetime component (Wendler and Holzwarth, 1987).

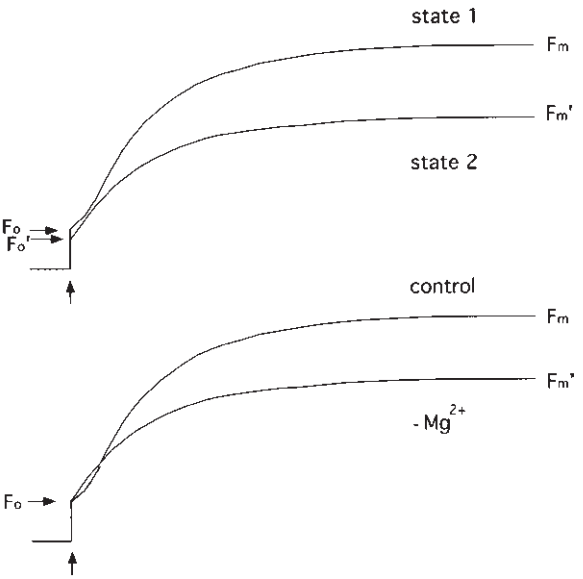

Fig. 4. Model fluorescence induction transients, illustrating the decrease in both F_m and F_o upon the transition to state 2 and in F_m alone on cation depletion ($-Mg^{2+}$). The upper transients suggest a decrease in the absorption cross-section of Photosystem II, the lower transients an increase in spillover from PS II to PS I. Such transients are obtained in the presence of DCMU. The y-axis is room-temperature fluorescence emission in arbitrary units, the x-axis is time on a millisecond scale. The rapid rise from the baseline to F_o occurs upon switching on the light (vertical arrow). Changes in sigmoidicity also occur. The $-Mg^{2+}$ and state 2 induction curves are markedly less sigmoidal than the controls.

B. Models for State Transitions, and Their Predicted Effects on Fluorescence Decay Kinetics

Two kinds of effects have been postulated to accompany state transitions:

(a) In the 'absorption cross-section' model, state transitions change the proportions of light-harvesting pigments connected to the two photosystems. This could most easily be accomplished by the detachment of a pigment-protein complex from one photosystem and its re-association with the other photosystem.

(b) In the 'spillover' model, energy transfer can occur between the PS II and PS I core complexes, and it is postulated that state transitions change the rate constant for this energy transfer. This could occur as a result of conformational changes that change the orientation of pigment molecules, or as a result

of an ultrastructural change that alters the spatial separation between PS II and PS I. As mentioned in Section I.A, PS I absorbs light at slightly longer wavelengths than PS II. More significantly, PS I is somewhat faster, a more efficient trap for excitation energy. This means that if energy transfer between PS II and PS I can occur, the net flow of excitation energy will generally be from PS II to PS I (Trissl and Wilhelm, 1993). Thus, a transition from state 1 to state 2 should involve an increase in the rate constant for spillover.

In principle, time-resolved fluorescence measurements can distinguish between the two models. Figure 5 shows, in a highly-simplified form, the predicted effects of spillover and absorption cross-section changes on decay-associated spectra, when the excitation is at a wavelength absorbed by the light-harvesting complex. The model assumes a simple system consisting of the two photosystems and a light-harvesting complex. Each complex has a characteristic fluorescence emission maximum and fluorescence decay lifetime.

In the absorption cross-section model the transition to state 2 involves the movement of a proportion of the light-harvesting complex from PS II to PS I (not

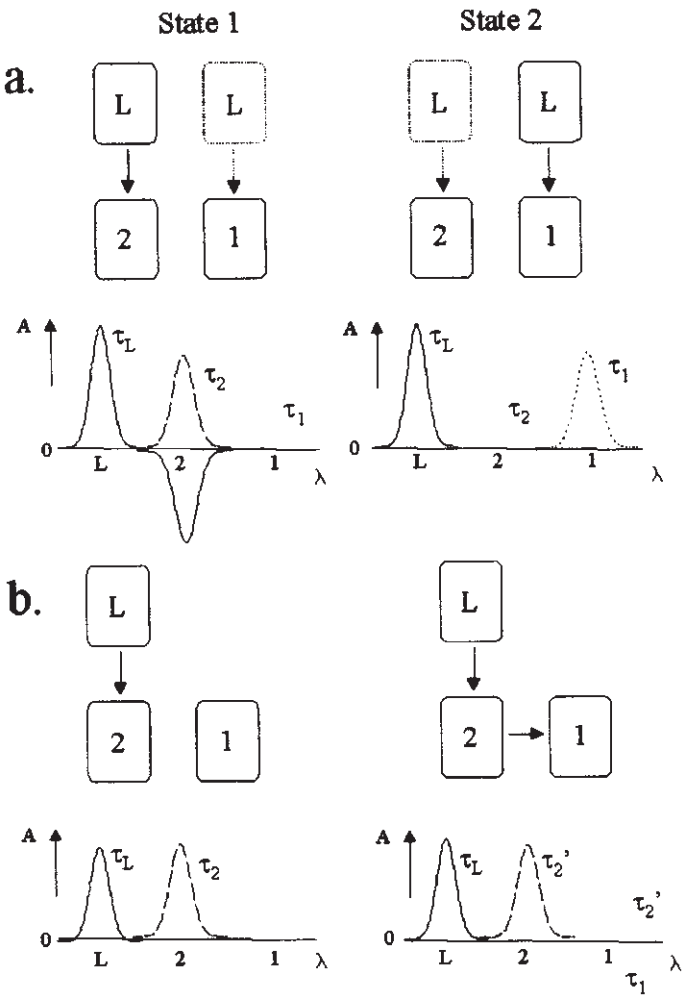

Fig. 5. Models for excitation energy distribution during state transitions, and their effects on decay-associated spectra. Both the models and the spectra are highly simplified and idealized. L: light-harvesting complex; 2: PS II; 1: PS I; A: fluorescence amplitude; τ: fluorescence lifetime. Negative amplitudes represent fluorescence rise-terms. (a) Absorption cross-section model. For the purposes of the figure it is assumed that the rate constants for energy transfer from L to state 1 and state 2 are similar. (b) Spillover model (assuming that state 1 decays much more rapidly than state 2).

apparent in Fig. 5a). This will change the fluorescence decay lifetime of the light-harvesting complex, if the rate constants for energy transfer to the two photosystems are different. For the sake of simplicity, Fig. 5a assumes the rate constants are the same. The main prediction of the absorption cross-section model is that the amplitude of the fluorescence decay from the two photosystems will change. Movement of the light-harvesting complex from PS II to PS I will decrease the amplitude of fluorescence emission from PS II, and increase that from PS I. However, the fluorescence decay lifetimes of the two reaction center core complexes will be unaffected.

In the spillover model (Fig. 5b) the state 2 transition will lead to a decrease in the fluorescence lifetime for PS II, without affecting the amplitude. The effects on PS I emission are complex. Because the fluorescence decay lifetime of PS I is faster than that of PS II, spillover will lead to a new PS I decay component with the same lifetime as the PS II decay.

In real systems, the effects of state transitions are more complex and harder to interpret than in the simple model shown in Fig. 5. The fluorescence emission from PS II, PS I and light-harvesting complexes usually overlaps, sometimes making the assignment of decay components to particular complexes problematic. Because of the dynamics of charge separation, PS II has at least two fluorescence decay lifetimes (Schatz et al, 1988). There may be heterogeneity in all the complexes, leading to many more fluorescence decay lifetimes (Roelofs et al, 1992). If the decay of PS I fluorescence is faster than energy transfer from the light-harvesting complex it will be difficult to resolve energy transfer from the light-harvesting complex to PS I. This may well be the case in cyanobacteria, where the lifetime for energy transfer from the phycobilisomes to the reaction center core complexes is around 180 ps (Mullineaux and Holzwarth, 1991), whereas the principal PS I fluorescence decay lifetime is 25 ps (Turconi et al., 1996).

C. Sample Preparation

Fluorescence lifetime measurements on intact systems generally involve collecting data over extended time periods. It is necessary to keep the sample in the appropriate light-state and to prevent the excitation light from perturbing or damaging the sample. Two approaches have been used:

(a) The sample is pre-adapted to the appropriate light-state and then rapidly frozen. Fluorescence decays are measured on the frozen sample, usually at liquid nitrogen temperature (77 K). This method has the additional advantage that fluorescence emission peaks are more sharply-defined. A problem is that cooling to 77 K may significantly alter the pathways of excitation energy transfer.

(b) The measurement is carried out at room temperature. A reservoir of sample is adapted to state 2 or state 1 using appropriate illumination conditions, and the sample is circulated through a flow-cuvette where it is exposed to the excitation light. Rapid circulation of the sample minimizes exposure to the excitation light. It is possible to use more complex flow systems to carry out measurements with open or closed PS II centers, as well as with adaptation to state 1 or state 2 (Mullineaux et al., 1990). Chloroplasts or membrane preparations may not be mechanically strong or stable enough for measurements under these conditions, so the measurements are generally carried out using intact algal or cyanobacterial cells.

D. Progress and Pitfalls of Using Time-resolved Fluorescence to Study State Transitions

There have been a number of studies using time-resolved fluorescence to probe state transitions in green algae (Wendler and Holzwarth, 1987) and phycobilisome-containing organisms (Bruce et al., 1985, 1986; Mullineaux et al., 1990). In cyanobacteria and red algae at 77 K, Bruce et al. (1985, 1986) found evidence for spillover changes. They reported a faster PS II fluorescence decay in State 2 (Bruce et al., 1985) accompanied by a slower rise of PS I fluorescence (Bruce et al., 1986). However, their analysis was based on the assumption that there is no significant overlap in the fluorescence emission from PS II, PS I and phycobilisomes. This is unlikely to be true, even at 77 K. Mullineaux et al. (1990) carried out measurements on cyanobacterial cells at room temperature. Decays were resolved into phycobilisome, PS II and PS I components using global data analysis. This study found no evidence for PS II lifetime changes. However, the amplitude of the PS II fluorescence decreased by about 60% in state 2, which would be consistent with the decoupling of about

60% of phycobilisomes from PS II. The closure of PS II reaction centers led to the appearance of long fluorescence lifetimes of about 1100 ps and 550 ps, which could be detected both in state 1 and in state 2 (Mullineaux and Holzwarth, 1991). These lifetimes are at least as long as those detected in isolated PS II core complexes (Schatz et al., 1988) which suggests that spillover to PS I is not a major pathway of energy transfer in cyanobacteria.

The main problem with the use of time-resolved fluorescence to probe physiological adaptation mechanisms is the extreme complexity of fluorescence decay kinetics in intact systems. There may simply be too many components to allow a rigorous analysis of the data. Until we are sure how many lifetimes are needed to describe fluorescence decays in intact systems, and where each lifetime component originates from, we may not be able to draw any definitive conclusions about adaptation mechanisms. Numerous mutants lacking reaction centers and/or light-harvesting complexes are now available, and a sensible approach would be to use these mutants as simplified systems for determining the kinetics of various energy transfer processes in vivo. We may then be able to go back to the wild-type system with more confidence.

IV. Using Fluorescence Recovery after Photobleaching (FRAP) to Study Protein Mobility

A. Protein Mobility and State Transitions

State transitions involve changes in the interactions of light-harvesting complexes and reaction centers. If we are to understand state transitions in physical terms, we need to know how the complexes can move in the membrane, and what movements are associated with state transitions. The problem has been approached in green plants by sub-fractionating thylakoid membranes into grana and stroma lamellae, and measuring the composition of the fractions (Staehelin and Arntzen, 1983). This technique can be used to demonstrate net migration of LHCII to the stroma upon adaptation to state 2, and to give a measure of the timescale of the migration (Drepper et al., 1993). In cyanobacteria, no such long-distance movements are postulated. However, the distribution of the complexes has been studied by freeze-fracture electron microscopy. The transition to state 2 seems to result in a decrease in the proportion of PS II reaction centers arranged in rows (Olive et al., 1986, 1997). These techniques do not give the whole story: they can be used to detect net changes in protein distribution upon adaptation, but they give little idea of the kinetics of the diffusion of complexes in the membrane. When using electron microscopy, some statistical pitfalls may arise when trying to extract quantitative data from a collection of images. In cyanobacteria, there is the additional problem that the phycobilisomes are not normally seen in freeze-fracture electron micrographs. An alternative approach is to use fluorescence microscopy and Fluorescence Recovery after Photobleaching (FRAP) exploiting the native fluorescence of the pigment-protein complexes. The spatial resolution of fluorescence measurements is obviously much lower than electron microscopy, but measurements can be carried out in vivo, allowing the migration of complexes to be monitored continuously. Progress with the technique, and the potential future developments, are discussed below.

B. Use of FRAP to Measure Protein Diffusion in Cyanobacteria

In cyanobacteria the PS II and PS I reaction centers are in close proximity in the membrane (Mullineaux, 1999). It is therefore unlikely that state transitions would result in any net migration of complexes over distances long enough to be resolved in fluorescence micrographs. However, FRAP can be used to monitor the mobility of the photosynthetic complexes (Mullineaux et al., 1997). In FRAP, a highly-focused confocal laser spot is used to bleach a small area of a cell or membrane, by photochemically destroying the chromophores. The recovery of fluorescence in the bleached area indicates diffusion of the pigment-protein complexes. Cyanobacteria are an excellent model system for FRAP because many species have a simple, regular thylakoid membrane organization. FRAP studies on the elongated cyanobacterium *Dactylococcopsis salina* showed that the phycobilisomes are extremely mobile, diffusing rapidly on the thylakoid membrane surface. However, the PS II core complexes do not diffuse at all (Mullineaux et al., 1997). Similar results are obtained in *Synechococcus* sp. PCC7942, where the diffusion coefficient for phycobilisomes at the growth temperature of 30 °C is about 3×10^{-10} cm^2 s^{-1} (Fig. 6). The phycobilisomes diffuse at a comparable rate in a mutant lacking the phycobilisome rods, which confirms that the FRAP

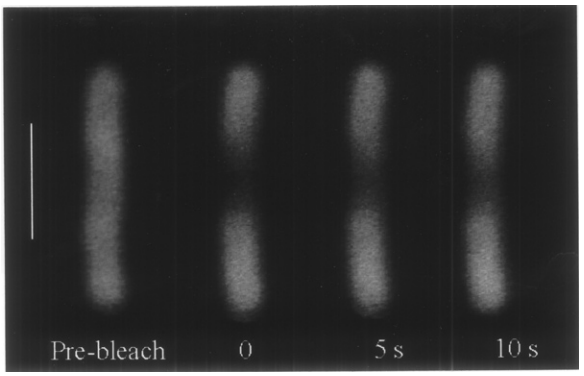

Fig. 6. FRAP(Fluorescence Recovery After Photobleaching) measurement of phycobilisome diffusion in a cell of the cyanobacterium *Synechococcus* 7942. The scale bar indicates 3 microns. Phycobilisome fluorescence was bleached by scanning a line across the centre of the cell, and the subsequent evolution of the bleaching pattern was monitored by recording images at 5 s intervals. Experimental conditions were similar to those described in Mullineaux et al., (1997).

studies are monitoring the diffusion of intact phycobilisomes rather than detached phycobilisome rods or free phycocyanin subunits (Sarcina et al., 2001). The fact that PS II is immobile, but phycobilisomes diffuse rapidly, implies that there is no stable phycobilisome-reaction center complex. Instead, it suggests that a phycobilisome will interact transiently with a reaction center, before decoupling, diffusing, and binding to another reaction center (Mullineaux et al., 1997; Mullineaux, 1999). Energy transfer studies indicate that phycobilisomes can interact with PS II as well as PS I reaction centers (Mullineaux, 1992, 1994). The distribution of phycobilisomes between PS II and PS I will therefore be governed by a dynamic equilibrium. The position of the equilibrium will be influenced by the relative concentrations of PS II, PS I and phycobilisomes, and the phycobilisome-PS II and phycobilisome-PS I binding energies.

C. FRAP and State Transitions in Cyanobacteria

The FRAP results described above do not give direct information on what happens during state transitions, but they give some clues:

a. The results suggest that phycobilisomes are a mobile element in state transitions. PS II appears to be completely immobile, at least over the relatively long distances that are monitored in a FRAP experiment. It remains possible that PS I mobility could play a role in state transitions (Schluchter et al., 1996). We do not yet have any direct information on PS I mobility, since PS I fluorescence is too low to be detected in a FRAP experiment. Tagging with Green Fluorescent Protein will allow us to monitor the movement of PS I and other non-fluorescent complexes. Note that it is likely that state transitions have more than one effect on light-harvesting. Mutagenesis studies indicate that there are phycobilisome-dependent and phycobilisome-independent effects (Olive et al., 1997; Emlyn-Jones et al., 1999).

b. It is important to note that phycobilisome diffusion occurs on a much faster timescale than state transitions. How long would it take for a phycobilisome to move from PS II to PS I? In cyanobacteria, PS II is typically arranged in parallel rows about 60 nm apart, with PS I located in the spaces between the rows (Olive et al., 1997). If we assume a typical PS II-PS I distance of 30 nm, and a phycobilisome diffusion coefficient of 3×10^{-10} cm^2 s^{-1}, then a phycobilisome could move from PS II to PS I in about 15 milliseconds. State transitions take place on a timescale of a few seconds to a few minutes (Fork and Satoh, 1983). It is therefore likely that the rate of state transitions is controlled by the rate of the signal transduction pathway, rather than by the rate of migration of the photosynthetic complexes. This also appears to be the case in green plants, where the kinetics of the state transition match the kinetics of LHCII phosphorylation (Bennett et al., 1980; Telfer et al., 1983).

c. The considerations above suggest the following model for state transitions in cyanobacteria. A change in the light environment changes the redox state of plastoquinone. This triggers an unknown signal transduction pathway which eventually leads to a change in the phycobilisome-PS II and/or phycobilisome-PS I binding energy. This shifts the equilibrium position so as to change the relative number of phycobilisomes coupled to PS II and PS I at steady-state. The phycobilisomes are mobile in both states — what changes is the statistical likelihood that phycobilisomes are coupled to PS II rather than PS I.

Could FRAP measurements be used to provide more direct information on the redistribution of protein complexes accompanying state transitions

in cyanobacteria? As discussed above, the spatial redistribution accompanying state transitions is likely to occur on a scale below 60 nm, far below the resolution of an optical measurement. However, the model above predicts that the diffusion coefficient for phycobilisomes should be different in state 1 and in state 2. It seems likely that the overall diffusion rate of the phycobilisomes is governed by their interaction with reaction centers. For example, the more stable the coupling between the phycobilisomes and the immobile PS II complexes, the less frequent will be the detachment of the phycobilisomes from PS II and the slower will be the diffusion of the phycobilisomes over the relatively long distances monitored in FRAP measurements. Thus, if the phycobilisome-reaction center binding constants change, then the diffusion coefficient for the phycobilisomes will also change. Unfortunately it may be very difficult to maintain cells in state 1 or state 2 during FRAP measurements. FRAP measurements in *Synechococcus* 7942 take place over a timescale of at least 20 s (Fig. 6), during which time significant adaptation can take place in cyanobacteria (Fork and Satoh, 1983). The measurement involves the exposure of the cell to intense light, both for the initial bleaching, and for the subsequent monitoring of the bleaching pattern. This light will certainly trigger state transitions, among other effects. In macroscopic measurements it is possible to monitor fluorescence using a measuring light too weak to perturb the state of the cells (Schreiber et al., 1995). This is not currently possible on a microscopic scale, where the excitation light must be intense enough to generate a measurable fluorescence signal from a single cell. A partial solution to the problem would be to use mutants 'trapped' in state 1 or state 2. A *Synechocystis* mutant which appears to be permanently in state 1 has been isolated (Emlyn-Jones et al., 1999). Similar specific mutants in *Synechococcus* 7942 would allow us to see if adaptation to state 1 or state 2 changes the mobility of the photosynthetic complexes.

D. Confocal Microscopy and FRAP in Green Plants

In green plants, the distribution of Chl-protein complexes between the grana and stroma lamellae can be assessed using confocal fluorescence microscopy (Gunning and Schwartz., 1999; Mehta et al., 1999). Potentially, it might be possible to use the technique for direct observation of LHCII migration between the grana and the stroma lamellae during state transitions. FRAP studies on chloroplasts will be harder than on cyanobacteria because of the convoluted structure of the thylakoid membrane (Sarafis, 1998). It may not be possible to do quantitative measurements except in some green algal chloroplasts that lack grana.

V. Screening for State Transition Mutants

The components of the signal transduction pathway that links changes in the redox state of the cytochrome b_6f complex to changes in light-harvesting are still largely unknown. Could a genetic approach be used to identify the signal transducers? Two approaches could be adopted:

a. Genome sequencing projects have revealed large numbers of genes coding for potential signal transducers (see Kotani and Tabata, 1998, for example). Knockout mutants for these genes could be screened for inability to perform state transitions. This approach has been unsuccessful so far. In the cyanobacterium *Synechocystis* 6803, many deletion mutants lacking genes for sensory histidine kinases have been screened: so far all have proved to be capable of performing state transitions (C.W. Mullineaux and A. Wilde, unpublished). It may be that state transitions do not operate through signal transducers sufficiently 'conventionally' to be recognized on the basis of sequence homologies.

b. The alternative approach is to generate libraries of random mutants, and then to screen for the inability to perform state transitions. This approach has proved to be more productive, and specific state transition mutants have been isolated in the cyanobacterium *Synechocystis* 6803 (Emlyn-Jones et al., 1999) and the green alga *Chlamydomonas reinhardtii* (Fleischmann et al., 1999; Kruse et al., 1999). In the case of *Synechocystis*, the gene involved has been identified. It codes for a putative membrane protein with no significant homology to any previously-characterized gene product (Emlyn-Jones et al., 1999).

If the approach described in (b) above is to succeed, it is necessary to have an efficient screening procedure. In *Synechocystis*, state transition mutants were identified at a frequency of about 1/4000 mutant

colonies (D. Emlyn-Jones and C.W. Mullineaux, unpublished). In organisms with larger genomes, many more mutants will have to be screened. Fluorescence video imaging provides an attractive way to screen for state transition mutants. State transitions result in a small change in the Chl fluorescence yield (typically 10–30%, depending on the organism). Therefore it is possible to use fluorescence video imaging to screen large numbers of cell colonies simultaneously. We routinely screen up to about 200 colonies simultaneously on a Petri dish. It is necessary to design the screening procedure carefully to avoid some pitfalls:

a. A random mutant library will contain many strains with different growth rates and different levels of Chl fluorescence. Therefore the simple approach of adapting the colonies to state 1 or to state 2 conditions and looking for colonies with abnormally high or low fluorescence will not provide a selective screen.

b. The obvious approach is therefore to record images for state 1-adapted colonies and state 2-adapted colonies, and to subtract the state 2 image from the state 1 image to obtain a difference image. This is not ideal, as the screen will be somewhat biased according to the background level of colony fluorescence. Weakly-fluorescent colonies will generally show a smaller fluorescence difference than strongly-fluorescent colonies, providing many potential 'false negatives.'

Figure 7 shows a screening strategy that avoids the pitfalls described above. The equipment required is a light source with a fiber-optic ring-light to distribute the light evenly on the Petri dish, cut-off filters, a computer-linked video camera and software for manipulating the images. We use Optimas 5.0 (Optimas Corporation). This software has the considerable advantage that it is easy to automate some of the image processing steps. The screen involves adapting the colonies to state 1 or to state 2 conditions and recording an image for each state. The state 1 image is then divided by the state 2 image to produce a ratio image. This avoids the pitfall described in (b) above. The ratio image shows the positions of all the colonies performing state transitions. Colonies not performing state transitions are invisible, as they have the same fluorescence ratio (1.0) as the background. The remaining steps in the procedure provide a way to highlight these colonies. First, the grey-scale of the ratio image is compressed above a threshold value, so as to produce a simple map showing the positions of all the colonies performing state transitions. One of the initial fluorescence images is treated in a similar way to produce a map showing the positions of all the colonies. Finally, the 'state transition map' is subtracted from the 'colony map' to produce a 'mutant map'. Any remaining white spots on the mutant map should indicate colonies which are *not* performing state transitions. The method illustrated in Fig. 7 has been used to isolate specific state transition mutants from *Synechocystis* 6803 (Emlyn-Jones et al., 1999) and *Chlamydomonas reinhardtii* (Kruse et al., 1999).

A similar approach could be used to screen *Arabidopsis* seedlings. For *Arabidopisis thaliana*, an independent fluorescence screening procedure designed to detect mutants unable to perform state transitions (Allen et al., 1995) reveals one phenotype produced by T-DNA insertion in a homologue of cyanobacterial *psbZ*, and 11 kDa protein associated with PS II (P. Davison, personal communication). The technique used (Fig. 8) allows visualization of Chl fluorescence during the full course of state transitions (as in Fig. 1) that are induced by addition of light 1 (710 nm) to a continuous, combined light 2 and excitation beam defined by a blue Corning 4–96 filter. Chl fluorescence is imaged by a red-extended CCD camera (Photonic Science ISIS) blocked by a 660 nm narrow-band interference filter. The imaged sequence was acquired, and processed by the program NIH-Image, using an Apple Macintosh computer. See also legend of Fig. 8.

VI. Concluding Remarks

What is required for a functionally useful description of the interactions of the components of photosynthetic membranes is not necessarily the increased resolution that can be provided by X-ray crystallography, and by the refinement of electron crystallography. Even low-resolution structural studies can be useful in revealing the supramolecular organization of biological membranes, although it is important to be able to identify the individual proteins securely. Perhaps future developments in providing a topographical map of native thylakoid membranes at subnanometer resolution and in different states (such as state 1 and state 2) can come from atomic

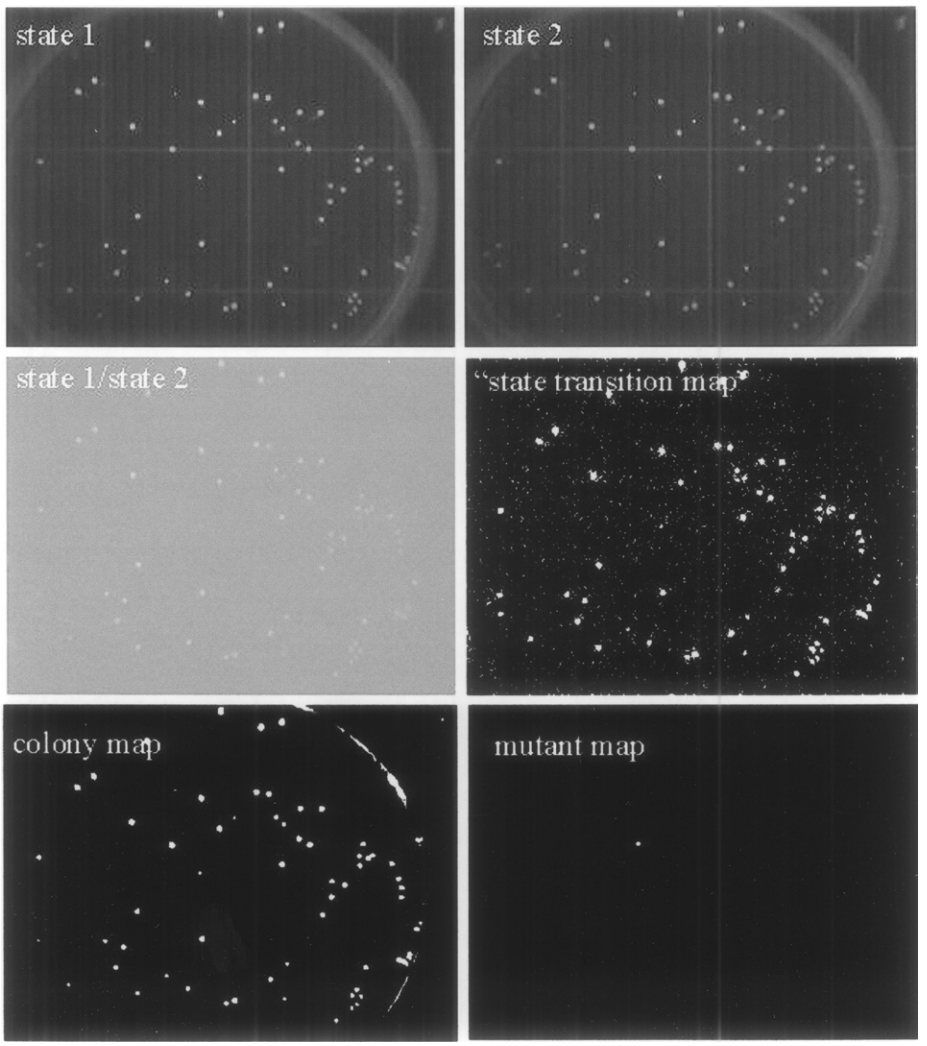

Fig. 7. Using fluorescence video imaging to screen for state transition mutants in microbes. In this case, the experimental organism is the cyanobacterium *Synechocystis* 6803. A collection of random mutants is plated out, and the video imaging system is set up to visualize Chl fluorescence from the colonies. Images of the colonies are recorded after adaptation to |the high fluorescence state 1 or to the low fluorescence state 2. The images are stored in the computer and digitally processed. First, the state 1 image is divided by the state 2 image to produce a ratio image (state 1/state 2). The grey-scale is then manipulated to produce a map showing the positions of all colonies in which the percentage fluorescence increase on transition to state 1 exceeds a certain threshold (state-transition map). The state 1 image is similarly manipulated to produce a map showing the positions of all the colonies (colony map). Finally, the state transition map is subtracted from the colony map to produce the 'mutant map'. Any remaining white spots are colonies which are not performing state transitions (Emlyn-Jones et al., 1999).

force microscopy. Such techniques could fill a gap in the scale of resolution that is created by the high resolution of protein structure determination and the low resolution of more traditional microscopy carried out on native membranes. Direct visualization of the movement of intrinsic membrane complexes relative to each other may be possible in high-resolution light and fluorescence microscopy, though, again, interpretation of what is moving relative to what will depend on inferences from biochemistry, biophysics, and structural biology. The antiquity of their origin, and the evolutionary continuity of state transitions, is indicated by their fundamental similarity in chloroplasts and in cyanobacteria. This perspective offers reasons for optimism about the possibility of unraveling the molecular details of thylakoid structure, function and dynamics — as well as those of the regulatory and signaling processes whose core features must surely,

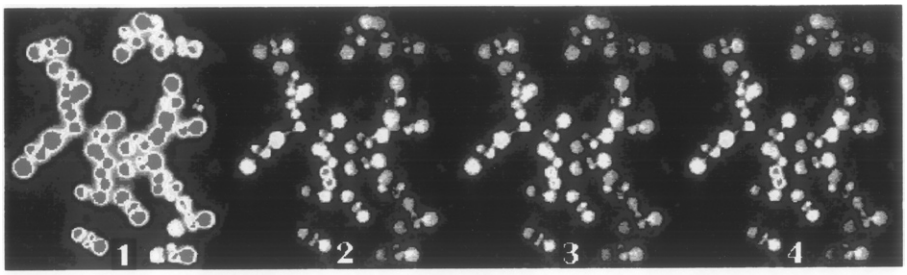

Fig 8. Using fluorescence video imaging to screen for state transitions in *Arabidopsis thaliana* plants. Fluorescence is initially high (seen in light grey color in image 1), and falls after six minutes in the light of combined excitation beam and light 2 (image 2), as LHC II becomes phosphorylated (giving state 2). Light 1, undetected by the camera, is switched on, and fluorescence falls as PS II traps open (image 3), to rise slightly after a further two minutes (image 4), as LHC II becomes dephosphorylated (giving state 1). One plant, whose two leaves are seen near the centre, behaves differently, and its fluorescence is consistently higher and less variable than that of others. The lighter the grey color the higher the fluorescence intensity at 660 nm. See also http://plantcell.lu.se/research/imaging.

too, have been conserved throughout the evolution of photosynthesis.

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