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Engineering of N-terminal threonines in the D1 protein impairs photosystem II energy transfer in *Synechocystis* 6803

Christiane Funk, Wolfgang P. Schröder, Gaza Salih, Ronney Wiklund, Christer Jansson*

Department of Biochemistry, The Arrhenius Laboratories, Stockholm University, S-10691 Stockholm, Sweden

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Abstract Mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 with N-terminal changes in the photosystem (PSII) II D1 protein were analysed by flash-induced oxygen evolution, chlorophyll *a* fluorescence decay kinetics and 77 K fluorescence emission spectra. The data presented here show that mutations of the Thr-2, Thr-3 and Thr-4 in D1 do not influence the oxygen evolution. A perturbation on the acceptor side was observed and the importance of the N-terminal threonines for an efficient energy transfer between the phycobilisome and PSII and for stability of the PSII complex was demonstrated.

Key words: D1; Photosystem II; *psbA*; Site-directed mutagenesis; *Synechocystis*

1. Introduction

Photosystem II (PSII) is an enzyme complex that couples light-induced charge separation with the reduction of plastoquinone and the oxidation of H_2O to O_2. PSII contains over 20 different polypeptide subunits (for reviews, see [1,2]). Two of these polypeptides, D1 and D2, with molecular weights of ~30 000, constitute the reaction centre core and harbour many of the key cofactors required for the primary photochemistry of PSII.

The D1 protein is encoded by the *psbA* gene. In cyanobacteria *psbA* belongs to a small multi-gene family with two to seven members [3]. In the cyanobacterium *Synechocystis* 6803, the *psbA* gene family contains three members: *psbA1*, *psbA2* and *psbA3* [3–7]. In chloroplasts, on the other hand, *psbA* exists as a unique gene on the plastid genome. The D1 and D2 proteins have evolved from the progenitors of the L and M subunits, respectively, of photosynthetic reaction centres in purple bacteria [8–10], and they are highly conserved [11,12]. Both the D1 and D2 polypeptides have five transmembrane helices each with their N-terminus at the stromal/cytosolic side and the C-terminus at the lumenal side.

In plants, turnover of the D1 protein is controlled by reversible phosphorylation [13–18] on N-terminal threonines, particularly Thr-2 [19] which is conserved in all but two of the over 40 different *psbA* open reading frames sequenced to date [11]. In the model by Rintamäki et al. [17], phosphorylation prevents proteolytic degradation of deactivated D1 protein in the grana stacks. After PSII core complexes with non-functional D1 protein have migrated to the stroma lamellae, the D1 protein is dephosphorylated and subsequently degraded.

No reversible D1 phosphorylation has been observed in cyanobacteria [19,20] although Thr-2 is conserved in all cyanobacterial *psbA* coding regions studied so far, and quite often is followed by a threonine also at position 3 [11]. In the cyanobacterium *Synechocystis* 6803, threonines occupy positions 2, 3 and 4 [11,12]. Site-directed mutagenesis in *Synechocystis* 6803 demonstrated that neither the conserved Thr-2 nor Thr-3 or Thr-4 are indispensable for photosynthetic growth [20]. However, engineering of the threonines to valine or leucine (mutant strains T2V;T3V;T4V, T2L;T4V and T2V) diminished O_2 evolution activity and slowed down growth. No effect on PSII assembly or D1 stability was observed in strain T2V;T3V;T4V under high light conditions and it was hypothesised that the impaired PSII activity could be due to altered interactions between the D1 N-terminus and other PSII polypeptides and/or the phycobilisome [20].

In the present investigation, we have analysed the N-terminal mutant strains of *Synechocystis* 6803 by measuring flash-induced oxygen evolution, chlorophyll *a* fluorescence decay kinetics, and 77 K fluorescence emission spectra, as means to further our understanding of the structural or functional significance of the N-terminal threonines in the D1 protein of *Synechocystis* 6803.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Wild-type and mutant strains of *Synechocystis* sp. PCC 6803 were cultured as described [4,20]. For all biophysical analyses, cells were harvested at a OD_{730} of 0.8–1.2 by centrifugation at 4000×*g* for 10 min. The cells were washed once in BG11 medium supplied with 20 mM HEPES/NaOH (pH 7.5), resuspended at a concentration of 0.5–0.8 mg chlorophyll/ml. The cells were placed in glass tubes and kept on a shaker at 30°C with a light intensity of 50 μE/m²/s. For fluorescence decay analyses, cell samples were treated one at a time and analyses were completed within 30 min.

2.2. Chlorophyll analysis

To determine the chlorophyll *a* concentration, 10 μl of cell suspension was mixed with 990 μl of methanol. The sample was centrifuged and the optical absorption of the supernatant at 663 nm was recorded and calculated according to Lichtenthaler et al. [21].

2.3. Measurements of O_2 evolution in continuous light

O_2 evolution was detected with a Clark-type electrode. Cells were suspended at a concentration of 10 μg/ml chlorophyll in BG11 supplemented with 20 mM HEPES/NaOH (pH 7.5). Measurements were performed at 25°C in the presence of 0.5 mM $K_3(Fe(CN)_6)$ and 0.1 mM 2,6-dichloro-*p*-benzoquinone (DCBQ) with yellow actinic light (570 nm cut-off filter) at a saturating intensity of 3000 μE/m²/s.

*Corresponding author. Fax: (46) (8) 153679.
E-mail: christer@biokemi.su.se

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Q_A and Q_B, primary and secondary plastoquinone electron acceptors

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2.4. Measurements of flash-induced O_2 evolution

Flash-induced O_2 oscillation patterns were measured with a modified Joliot-type electrode [22]. The flow medium contained 50 mM HEPES/NaOH (pH 7.0) and 30 mM $CaCl_2$ and was kept at 25°C. The samples were dark-adapted on the platinum electrode for 3 min. The polarisation voltage of −750 mV was switched on 60 s before excitation with a train of short (10 μs), saturating Xe flashes, separated by a dark time of 500 ms. The flash-induced O_2 yields (Y_n) were measured and normalised to the average of the yield obtained on flash 3, 4, 5 and 6, here defined as the steady-state value, Y_{ss}.

2.5. 77 K fluorescence emission analyses

77 K fluorescence emission spectra were recorded on a Perkin-Elmer luminescence spectrometer. The cells were frozen in BG11 plus 20 mM HEPES/NaOH (pH 7.5) without glycerol to prevent disruption of the phycobilisomes. Excitation was performed at 440 nm for chlorophyll excitation or 590 nm for phycobilin excitation. Each sample had a chlorophyll concentration of 5 μg/ml. Spectra were corrected for wavelength-dependent sensitivity of the photodetector.

2.6. Fluorescence decay analyses

Fluorescence decay measurements were performed after a single saturating flash with a home-built (flash probe) modulated LED fluorimeter [23]. Concentrated cell cultures were diluted with BG11 plus HEPES/NaOH (pH 7.5) to a total chlorophyll concentration of 40 μg. For measurements of Q_A^- charge recombination kinetics after a flash, the samples were incubated in darkness for 1 and 5 min, respectively, after addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a concentration of 40 μM. The weak monitoring flashes (10 kHz), provided by a 625 nm LED array, filtered with a LS-650 filter, were switched on and the saturating actinic flash was applied approximately 30 s later. Fluorescence was detected by a photodiode/RG-715 filter combination. The actinic flash was supplied by a xenon flash lamp (3 ms half-width) filtered through a Schott BG-18 filter. Variable probe modulation was controlled by the data acquisition software.

3. Results

Previously we have described [20] the construction of *Synechocystis* strains with the N-terminal threonines of the D1 protein replaced by valines or leucines. Three of these mutant strains, the triple mutant T2V;T3V;T4V, and the single mutants T2V and T2L, were used in the present study.

3.1. Oxygen evolution under continuous light

Table 1 summarises the average results of three electron transport measurements made in whole cells of the different strains. Strains T2V and T2L exhibited only slightly lower DCBQ-supported O_2 evolution rates compared to the wild type, whereas for strain T2V;T3V;T4V, a slow initial electron transport rate was observed, corroborating the results from our initial characterisation of these mutants [20]. Interestingly, while the wild type and strains T2V and T2L showed stable O_2 evolution for more than 10 minutes at 3000 μE/m^2/s, in strain T2V;T3V;T4V, O_2 evolution ceased within 2 min. This short-lived O_2 evolving capacity in T2V;T3V;T4V could be indicative of either destabilisation of the water-oxidising complex, or photoinhibition within PSII.

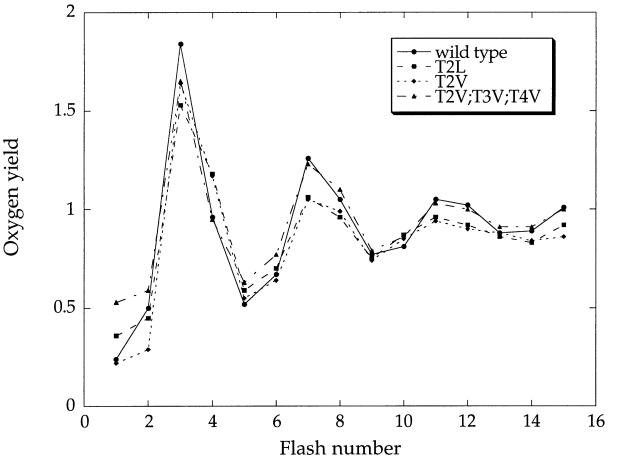

Fig. 1. Oxygen yield pattern in dark-adapted wild-type and mutant strains of *Synechocystis* 6803, detected with a Joliot-type electrode after illumination with a train of 15 flashes.

3.2. Flash-induced O_2 evolution

To study the integrity of the water-oxidising complex in the three mutants, we compared the flash-induced O_2 evolution in wild-type and mutant strains. The O_2-oscillation patterns of intact cyanobacterial cells exhibit some differences compared to those of higher plant thylakoids. Excitation of dark-adapted cyanobacterial cells with a flash train led to a signal pattern with O_2 evolution already on the first two flashes [24,25]. The presence and size of this polarographic response on the first two flashes seemed to be dependent on the growth state and age of the cell cultures. Whereas young cultures (OD_{730} = 0.3–0.6) of wild-type and mutant strains exhibited a higher O_2 evolution on the first two flashes, older cultures (OD_{730} = 0.8–1.2) did not show this distinct feature (data not shown). However, it is not certain whether the response caused by the first flash is related to O_2 formation or some other physiological conditions within the cell or thylakoid [25]. We were not able to abolish the O_2 yields on the first two flashes by preflashes [25], by adding catalase [26], or by increased incubation times on the electrode.

The normalised flash patterns for wild-type and mutant strains are shown in Fig. 1. All strains showed a similar flash pattern with a period of four, and no difference in damping could be observed. Consequently, and as would be expected from mutagenesis of the cytosolic/stromal N-terminal of the D1 protein, the water oxidase machinery of PSII was unaffected in the mutant strains.

3.3. Charge recombination between Q_A^- and $P680^+$

Normally, Q_A^- reoxidation is dominated by the forward electron transfer reaction to the secondary quinone acceptor Q_B ($t_{1/2}$ = 100–200 μs [27]), but for samples with DCMU

Table 1
Oxygen evolution rates and photodamaging times in wild-type and mutant strains of *Synechocystis* 6803[a]

Strain	O_2 evolution rate (μmol O_2/mg Chl/s)	Activity of wild type (%)	Photodamaging time
Wild type	436	100	–
T2V	324	74 ± 11	–
T2L	277	64 ± 8	–
T2V;T3V;T4V	245	56 ± 9	2.5 min

[a]Measurements represent an average from three different cultures.

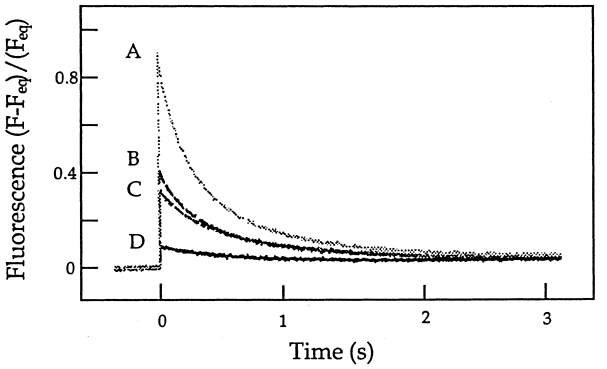

Fig. 2. Formation and decay of Q_A^- in response to a saturating flash in *Synechocystis* 6803 wild type (A) or mutant strains (T2L (B), T2V (C) or T2V;T3V;T4V (D)), as measured by changes in the yield of chlorophyll *a* fluorescence. Measurements were performed in the presence of DCMU.

present, where the forward reaction to Q_B is blocked, the rate of Q_A^- oxidation becomes dependent on recombination reactions. In *Synechocystis* 6803 the yield of variable chlorophyll *a* fluorescence is believed to reflect the amount of Q_A^- in the sample [28] and thus this type of measurements is often used as a rapid method for quantifying PSII content [29]. The same amount of chlorophyll (40 μg) was applied for all samples and as is evident in Fig. 2, all mutants showed a decreased fluorescence induction compared to wild type. Strains T2L and T2V showed approximately 50%, and strain T2V;T3V;T4V more than 90%, reduced chlorophyll *a* fluorescence induction. On the face of it, this would suggest a severe reduction in the amount of Q_A^-, and hence PSII complexes, in the mutants. However, all mutants exhibited roughly normal oxygen evolving rates (Table 1), normal oxygen yield pattern (Fig. 1) and close to wild-type levels of the D1 protein [20]. Thus we interpret the reduced amount of Q_A^- in the mutant strains to reflect decreased stability of Q_A^- due to incomplete assembly of the cofactors involved in the electron transfer within PSII.

The decay kinetics from chlorophyll *a* fluorescence measurements is dependent not only on the charge recombination between Q_A^- and P_{680}^+ but also the equilibrium concentration of P_{680}^+. A slower recombination kinetics would thus reflect changes on the donor side of P680, preferentially in the Y_z or *s* states. Due to the overall decreased chlorophyll *a* fluorescence in the mutants a reliable decay analysis could not be performed. However, a rough comparison between the wild type and the mutants did not reveal any significant changes in decay half times, which shows that no drastic changes had occurred on the donor side of PSII. This conclusion was also supported by the oxygen yield and rate data (Table 1, Fig. 1).

3.4. 77 K fluorescence emission

Measurements of 77 K fluorescence emission spectra provide a sensitive way to monitor the energy transfer and presence of PSII. Fig. 3 shows the 77 K fluorescence emission spectra of the wild type and the T2V, T2L and T2V;T3V;T4V strains. Upon excitation at 440 nm, which excites chlorophyll *a*, three peaks at 686, 695 and 725 nm could be seen (Fig. 3A). The main peak at 725 nm corresponds to PSI, whereas the two peaks at 685 nm and 695 nm are linked to PSII. The peak at 685 nm reflects all chlorophylls associated with PSII (including chlorophyll from CP43, D2, D1 and CP47), except for one low-energy chlorophyll that appears to be associated with His-114 of CP47. This low-energy chlorophyll is the main contributor to the 695 nm emission maximum [30].

All fluorescence emission spectra were normalised at 725 nm. At an excitation of 440 nm the strains T2L and T2V

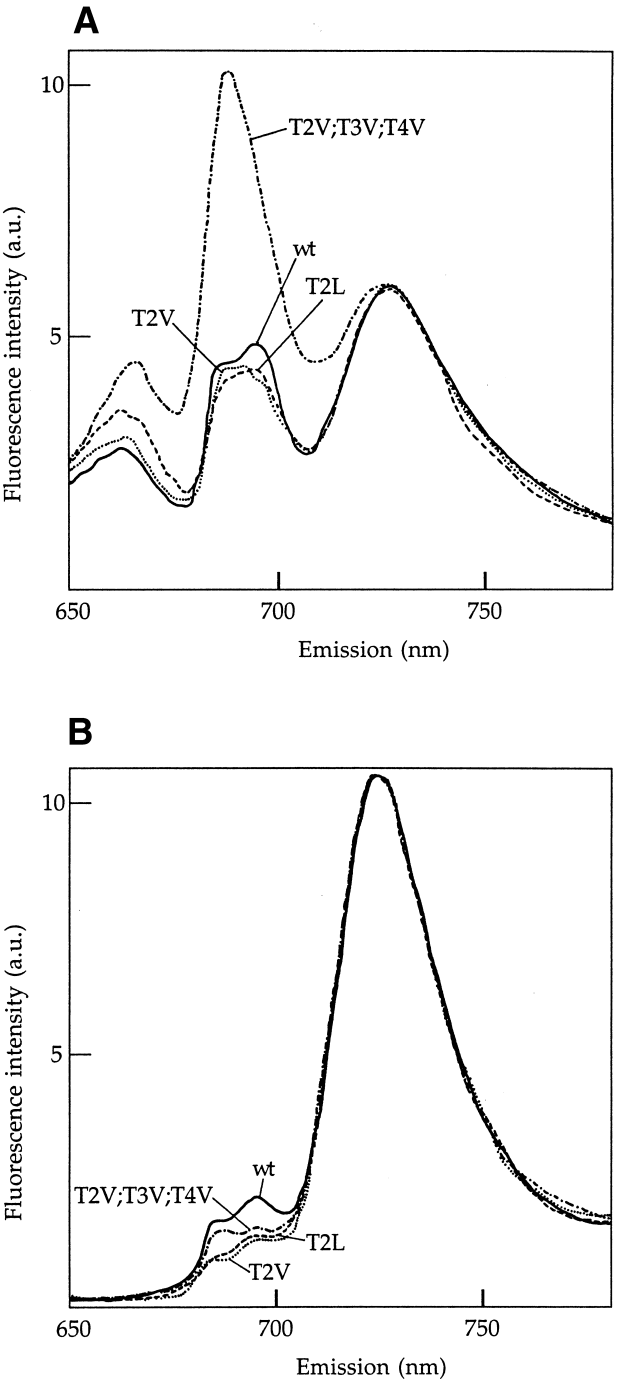

Fig. 3. Fluorescence emission spectra at 77 K from whole cells of *Synechocystis* 6803 wild type and mutant strains T2L, T2V and T2V;T3V;T4V with an excitation wavelength of 440 (A) or 590 (B) nm. The spectra were normalised at the maximum (725 nm in A and B). No glycerol was added to the cells in order to prevent disruption of the phycobilisomes.

exhibited almost the same fluorescence spectrum as the wild type, indicating that both PSI and PSII were properly assembled and fully functional (Fig. 3A). Also strain T2V;T3V;T4V produced the three fluorescence maxima at 685 nm, 695 nm and 725 nm but the 695/685 ratio was decreased, suggesting that the PSII energy transfer was impaired.

Even more dramatic effects were obtained after excitation at 590 nm, which excites allophycocyanin in *Synechocystis* 6803 (Fig. 3B). The cells were frozen without glycerol to avoid functional uncoupling of the phycobilisomes from thylakoid components. The spectra were normalised at 725 nm. The maximum seen in the 77 K fluorescence emission spectra at 660 nm originates from phycobilisome components (phycocyanin and allophycocyanin, respectively) [31]. The peak at 695 nm with a shoulder at 685 nm originates from PSII, and the maximum at 725 nm from PSI. In the wild type, the phycobilin fluorescence exhibited the smallest maximum, and PSI the biggest. In strains T2L and T2V, the energy transfer was only marginally affected. The slightly higher fluorescence observed in the T2L strain may be due to disconnection of some of the phycobilisomes. This might also explain the higher fluorescence at 685 nm, originating from allophycocyanin B and chlorophyll *a* in PSII. In the T2V;T3V;T4V strain, however, the phycobilisome fluorescence was even higher and in the 685–695 nm region only a single peak (at 685 nm) was detected, also originating from allophycocyanin and the Chl *a* of PSII. Thus the energy transfer from the phycobilisomes to PSII and further to PSI seem perturbed.

4. Discussion

We have previously demonstrated that engineering of the N-terminal threonines in the PSII reaction centre protein D1 slowed down photosynthetic growth and oxygen evolution in the cyanobacterium *Synechocystis* 6803, without affecting the amount or stability of the D1 protein in the thylakoid membrane [20]. In this study, we provide results suggesting that the reduced activities of the mutant strains can be attributed to impaired energy transfer to, and within, PSII. As is evident from Fig. 3B, fluorescence from the phycobilisomes is significantly increased in the mutants, particularly in strain T2V;T3V;T4V, where all three N-terminal threonines have been replaced with valines. This could indicate a functional uncoupling of the phycobilisome from PSII. Since the structural coupling between the terminal emitter of the phycobilisome and PSII is thought to be via CP47 [32] and possibly CP43 [32,33], it is not immediately obvious why alterations in the N-terminal arm of D1 would affect the association between the phycobilisome and PSII, if there is not a direct energy transport from phycobilisomes to D1. However, it is quite conceivable that a conformational change of the D1 N-terminus, invoked by the mutations [20], could perturb the integrity of the PSII reaction centre, including CP47, at the cytosolic surface of the thylakoid membrane. Indeed, if the fluorescence peak at 695 nm mainly originates from a CP47-associated chlorophyll [30], the emission spectra for strain T2V;T3V:T4V in Fig. 3A,B are indicative of a functional lesion in CP47 performance. Thus our data suggest a scenario where mutagenesis of the N-terminal threonines in D1 decreases the efficiency of energy transfer from the phycobilisome to PSII, resulting in a reduction in the oxygen evolving capacity (Table 1). The mechanistic properties of the oxygen evolving machinery, however, are clearly unaffected (Fig. 1).

Our own studies on *Synechocystis* 6803, and the work by Heifetz et al. [34] on *Chlamydomonas reinhardtii* strongly suggest that the conserved Thr-2 in D1 is not subjected to reversible phosphorylation and is not involved in D1 degradation in these organisms. Recently the same interpretation was made for D2 in *Chlamydomonas reinhardtii* [35]. According to the model by Rintamäki et al. [17], phosphorylation prevents proteolytic degradation of D1 in the grana stacks. Following migration of PSII core complexes with deactivated D1 to the stroma lamellae, the D1 protein is dephosphorylated and subsequently degraded. We hypothesised [20] that since thylakoid membranes in cyanobacteria do not exhibit a lateral heterogeneity, a protective mechanism of damaged D1 based on lateral migration, and hence reversible phosphorylation, might not be required in cyanobacteria. In this context, it is interesting to note that also in *Chlamydomonas reinhardtii*, there is no strict differentiation between grana and stroma lamellae. Although our results do not yield conclusive evidence against D1 phosphorylation in *Synechocystis* 6803, the data presented here indicate, that in cyanobacteria, the presence of threonines at the N-terminal arm of D1 is of structural, rather than functional, importance.

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References

[1] Vermaas, W.F.J., Styring, S., Schröder, W.P. and Andersson, B. (1993) Photosynth. Res. 38, 249–263.
[2] Pakrasi, H. (1995) Annu. Rev. Genet. 29, 755–776.
[3] Mohamed, A., Eriksson, J., Osiewacz, H.D. and Jansson, C. (1993) Mol. Gen. Genet. 238, 161–168.
[4] Jansson, C., Debus, R., Osiewacz, H.D., Gurevitz, M. and McIntosh, L. (1987) Plant Physiol. 85, 1021–1025.
[5] Mohamed, A. and Jansson, C. (1989) Plant Mol. Biol. 13, 693–700.
[6] Mohamed, A. and Jansson, C. (1989) in: Current Research in Photosynthesis (Baltscheffsky, M., Ed.), Vol. III, pp. 565–568, Kluwer Academic, Dordrecht.
[7] Mohamed, A. and Jansson, C. (1991) Plant Mol. Biol. 16, 891–897.
[8] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[9] Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.
[10] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[11] Jansson, C. and Mäenpää, P. (1997) in: Progress in Botany (Esser, K., Ed.), Vol. 58, pp. 352–367, Springer-Verlag, Heidelberg.
[12] Svensson, B., Vass, I. and Styring, S. (1991) Z. Naturforsch. 46c, 765–776.
[13] Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1143, 113–134.
[14] Elich, T.E., Edelman, M. and Mattoo, A.K. (1992) J. Biol. Chem. 267, 3523–3529.
[15] Elich, T.E., Edelman, M. and Mattoo, A.K. (1993) EMBO J. 12, 4857–4862.
[16] Mattoo, A.K., Marder, J.B. and Edelman, M. (1989) Cell 56, 241–246.
[17] Rintamäki, E., Kettunen, R. and Aro, E.-M. (1996) J. Biol. Chem. 271, 14870–14875.
[18] Rintamäki, E., Kettunen, R., Tyystjärvi, E. and Aro, E.-M. (1995) Physiol. Plant. 93, 191–195.
[19] Allen, J.F. (1992) Biochim. Biophys. Acta 1098, 275–335.
[20] Salih, G. and Jansson, C. (1998) Plant Mol. Biol. 36, 585–591.

[21] Lichtenthaler, H.K., Buschmann, C., Rinderle, U. and Schmuck, G. (1986) Radiat. Environ. Biophys. 25, 297–308.
[22] Joliot, P. (1972) Methods Enzymol. 24, 123–134.
[23] Ghirardi, M.L., Lutton, T.W. and Seibert, M. (1996) Biochemistry 35, 1820–1828.
[24] Bader, K.P. (1994) Biochim. Biophys. Acta 1188, 213–219.
[25] Gleiter, H.M., Haag, E., Shen, J.-R., Eaton-Rye, J.J., Inoue, Y., Vermaas, W.F.J. and Renger, G. (1994) Biochemistry 33, 12063–12071.
[26] Schröder, W.P. and Åkerlund, H.-E. (1986) Biochim. Biophys. Acta, 359–363.
[27] Robinson, H.H. and Crofts, A.R. (1983) FEBS Lett. 153, 221–226.
[28] Philbrick, J.B., Diner, B.A. and Zilinskas, B.A. (1991) J. Biol. Chem. 266, 13370–13376.
[29] Chu, H.A., Nguyen, A.P. and Debus, R.J. (1994) Biochemistry 33, 6137–6149.
[30] Shen, G. and Vermaas, W.F.J. (1994) Biochemistry 33, 7379–7388.
[31] Shen, G., Boussiba, S. and Vermaas, W.F.J. (1993) Plant Cell 4, 1853–1863.
[32] Bald, D., Kruip, J. and Rögner, M. (1996) Photosynth. Res. 49, 103–118.
[33] Nilsson, F., Simpson, D.J., Jansson, C. and Andersson, B. (1992) Arch. Biochem. Biophys. 295, 340–347.
[34] Heifetz, P.B., Lers, A., Boynton, J.E., Gilham, N.W. and Osmond, B. (1992) in: Research in Photosynthsis (Murata, Ed.), Vol. III, pp. 417–420, Kluwer Academic, Dordrecht.
[35] Andronis, C., Kruse, O., Deàk, Z., Vass, I., Diner, B.A. and Nixon, P.J. (1998) Plant Physiol. 117, 515–524.