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Comparative studies on electron transfer in Photosystem II of herbicide-resistant mutants from different organisms

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We have studied the electron transfer properties of Photosystem II using several techniques (fluorescence, oxygen emission and thermoluminescence measurements) in a series of herbicide-resistant mutants from widely different organisms. Five mutants of *Synechocystis* 6714, of which we have determined the D1 sequence, one mutant of *Synechococcus* 7942, one mutant of *Chlamydomonas reinhardtii*, a triazine-resistant biotype of *Chenopodium album* and their herbicide-susceptible controls were analyzed. Two mutants have an almost unimpaired Photosystem II electron transfer. For five mutants of the different organisms, the initial phase of the electron transfer Q_A^- to Q_B is unaltered but the electron transfer equilibrium between these two acceptors is displaced. In the *Chlamydomonas*-resistant mutant, the electron transfer from Q_A^- to Q_B is slowed down.

Introduction

Different classes of herbicides block the electron transfer between the primary (Q_A) and the secondary (Q_B) electron acceptor of Photosystem II (PS II) [1]. The herbicides and the Q_B quinone bind in the same region of the D1 protein [2], which is part of the PS II core complex. For several herbicide-resistant mutants selected in higher platns, green algae and cyanobacteria, the sequence of the *psbA* gene coding for D1 is known [3–10]. These forms of resistance are due to a drastic decrease in the affinity constant of the herbicide for its binding site, produced by a mutation of the D1 protein.

The most frequent mutation site in the D1 protein is at serine-264. In the three resistant mutants of higher plants which have been sequenced, serine-264 is replaced by a glycine [8,11,12], whilst it is replaced by an alanine in the DCMU-4 mutant of *Chlamydomonas reinhardtii*, in the Taq 1 mutant of *Synechococcus* 7942 and in the DCMU IIA mutant of *Synechocystis* [5,9,13]. Other mutation sites have been characterized in our laboratory for *Synechocystis* 6714 [10,13,14]. Each mutation is correlated with a specific pattern of cross-resistance to different classes of herbicides (see Table I).

The first results published on electron transfer in herbicide-resistant thylakoids showed that the Q_A^- to Q_B electron transfer was at least 10-times slower than in the susceptible thylakoids [15,16]. It was shown later that, in the herbicide-resistant mutant, the damping of flash-induced oxygen emission was increased [17], the deactivation of S_2 was faster [17,18] and the thermoluminescence peak corresponding to $S_2Q_B^-$ back-reaction was shifted to lower temperatures [19]. All these changes are consistent with an increased quasi-steady-state Q_A^- concentration due to a decreased equilibrium constant for the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$.

Using resistant mutants of cyanobacteria, our group and Robinson et al. [22,23] showed that the increased steady-state concentration of Q_A^- was not always linked to a much slower Q_A^- reoxidation.

More recently, Erickson et al. [24] showed that, in *C. reinhardtii*, only two out of five herbicide-resistant mutants showed a significant decrease in the rate of Q_A^- reoxidation.

Here, we have undertaken a comparative study of electron transfer in mutants of different organisms. We used different techniques (fluorescence, oxygen polarography, thermoluminescence) to obtain a more complete set of data than has been previously published. Our experimental approach was to first check whether the mutation inducing herbicide resistance invariably resulted in an increased amount of Q_A^- under steady-state conditions. We then checked whether the increased steady-state concentration of Q_A^- could be correlated to a decreased rate of Q_A^- reoxidation by Q_B.

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TABLE I

Mutations and relative herbicide resistance of the various species studied

Strain		Change in D_1	Relative resistance			Ref.
			DCMU	atrazine	ioxynil	
Synechocystis 6714	DCMU-IIA	264 Ser → Ala	500	70	1	13
	DCMU-IIB	264 Ser → Ala 255 Phe → Leu	1000	1	1	13
	AzI	211 Phe → Ser	1	10	–	14
	AzV	{ 251 Ala → Val 211 Phe → Ser }	2	100	–	13
	IoxI	266 Asn → Thr	0.8	1	10	10
Synechococcus 7942	Taq 1	264 Ser → Ala	100	10	–	9
Chlamydomonas reinhardtii	D4	264 Ser → Ala	10	100	–	5
Chenopodium album	R	264 Ser → Gly	1	500	–	8

With the data obtained, we discussed the effects of specific amino-acid substitutions in D1 on herbicide resistance patterns and electron transfer properties for different organisms.

Material and Methods

Material and growth conditions

Synechocystis 6714 wild type or mutants isolated and characterized in our laboratory [6,10,13,14,22] and *Synechococcus* 7942 or its mutant Taq 1 [23] were grown as in [22].

C. reinhardtii wild type and DCMU-4 mutant described in Ref. 5 were grown in continuous dim light in Tris-acetate-phosphate medium.

Chenopodium album S and R biotypes, described in Ref. 7 were grown in a local green-house (Phytotron, Gif sur Yvette). Thylakoids were isolated as in Ref. 25 except for the pH of the measurement medium, which was 6.5 instead of 7.8.

Fluorescence measurements

The fluorescence decay after a short saturating flash was measured in an apparatus already described [26]. A pulse-light-emitting diode (645 nm) was used as a non-actinic detecting beam (the pulses were 2 μs, spaced at 16 μs). The set of filters was a 4-96 Corning filter in front of the short saturating flash and a combination of a KV 550 (Schott), a RG 5 and an interference filter centered at 685 nm in front of the photomultiplier tube (S 20 light sensitivity). Under these conditions, less blue light falls on the RG 5 and the residual luminescence of the RG 5 filter is cut off by the interference filter.

The material was dark adapted for at least 10 min and the sample was renewed before each recording. To get a good signal-to-noise ratio, the curve was averaged 20 times.

Oxygen measurements

The amount of oxygen produced by each flash of a sequence of short saturating flashes evenly spaced was measured with apparatus previously described [27]. The parameters of the 'S cycle' as defined by Kok were computed by the matrix analysis developed by Lavorel [28]. The deactivations of S2 and S3 were studied by varying the dark time after one or two flashes, following a procedure already described [29].

Thermoluminescence

The thermoluminescence cuvette, 1 mm thick, was formed by a rubber plate with a 1×2 cm cavity, pressed between a plexiglass window and an aluminium plate which could be dipped partly or totally in liquid nitrogen. A 'Thermocoax' heater, on the other side of the plate, was used for regulation of temperature which was measured in the cuvette by a thermocouple.

Samples were prepared just before each measurement, by centrifugation of cell cultures and resuspension at 400 μg chlorophyll/ml (in the presence of 30% glycerol for *Chlamydomonas* cells). After 5 min in the dark at 20 °C followed by a slow cooling, a saturating flash was given at −5 °C and the sample was rapidly frozen. After 30 s temperature equilibration at −40 °C, the temperature was linearly increased to +80 °C in 4 min (0.5 C°/s). The luminescence emission was measured, at wavelengths above 650 nm, by a cooled photomultiplier connected to a photon counting system. Signal recording and temperature regulation were performed by an HP 85 microcomputer.

Results

Oxygen sequences

The oscillations of the amount of oxygen produced per flash, during a sequenc of flashes spaced at 500 ms,

TABLE II

Electron transfer characteristics of the various strains

		Fluorescence decline (phase amplitude, %)			Oxygen thermoluminescence	
		fast	medium	slow	misses	peak B, t (°C) (±1.5°)
Synechocystis	WT	60	20	20	0.097	38.0
6714	DCMU-IIA	42	20	38	0.160	29.7
	DCMU-IIB	48	16	36	0.146	31.0
	AZI	56	17	25	0.110	39.7
	AzV	42	23	35	0.140	32.6
	IoxI	55	26	19	0.108	41.2
Synechococcus	WT	58	16	26	–	–
7942	Taq 1	34	27	39	–	–
C. reinhardtii	WT	60	20	20	0.140	26
	D4	30	30	40	0.140	22
C. album	S	70	15	12	0.075	35 [a]
	R	65	10	24	0.124	18 [a]

[a] Measured on leaves.

are damped because of double hits and misses [28]. The reopening of centers was completed during the 0.5 s spacing between flashes, which is short compared to the half-times of S_2 and S_3 deactivation.

An increase of Q_A^- concentration remaining 500 ms after the preceding flash will increase the miss parameter. The calculated miss parameter (Table II) was significantly increased for *C. album*-resistant thylakoids and for *Synechocystis* DCMU-IIA, DCMU-IIB and AzV mutants. The increase was small for *Synechocystis* AzI and IoxI mutants. No increase was detected for the *C. reinhardtii* DCMU-4 mutant.

Deactivation kinetics

When a negative charge is stored on the acceptor side, the electron is shared between Q_A and Q_B. If the probability for the electron to be on Q_A is increased, there will be a concomitant increase in the rate of back reaction with the positive charge stored on the donor side. This rate was studied by S_2 deactivation kinetics followed by oxygen emission. In atrazine-resistant *C. album* thylakoids, the S_2 decay was faster than in atrazine-susceptible thylakoids (equivalent results were reported by Vermaas [18] for *Brassica napus*).

In *Synechocystis,* deactivation of S_2 for the IoxI mutant was the same as for the wild type. It was twice as fast for the DCMU-IIA mutant (at 29°C, the half-time of S_2 deactivation was 13 s for the wild type, 7 s for the DCMU IIA mutant).

In *C. reinhardtii,* the kinetics of S2 deactivation are similar for the DCMU-4 mutant and for the wild type (the half-time of deactivation was approx. 13 s in both cases).

Thermoluminescence emission

Charge recombination gives rise to luminescence, or thermoluminescence when the sample is progressively heated after a low-temperature illumination [30]. In the glow curve of chloroplasts, the main thermoluminescence band appearing at about +30°C can be attributed to the $S_2Q_B^-$ back-reaction (B band) [30,31].

In a previous study, it was shown that the peak position of the B band was shifted from 32°C to 15°C of resistant chloroplasts compared to the sensitive chloroplasts of *Erigeron canadensis* L. [19]. We obtained similar data with leaves of triazine-resistant and -susceptible *C. album* (Table II). The shift of the B band in *Synechocystis* was different for each mutant. The largest shift was seen from DCMU-IIA. The B band of AzI and IoxI was not different from that of wild type. An increase of luminescence emission in the Q band region ($S_2Q_A^-$ recombination) could be detected in these mutants from the first derivative of the thermoluminescence signal, but the overlap with the low-temperature-shifted B band prevented an exact determination.

In *Chlamydomonas* DCMU-4, the shift was small (Table II).

Fluorescence decay after a saturating flash

The decay of chlorophyll fluorescence at short times following flash illumination reflects the kinetics of reoxidation of the primary acceptor, Q_A^-, by Q_B. At longer times, the variable fluorescence and its kinetics reflect the concentration of Q_A^- in equilibrium with Q_B^- and some variable fluorescence will remain as long as Q_B^- is not completely reoxidized. It is also possible that

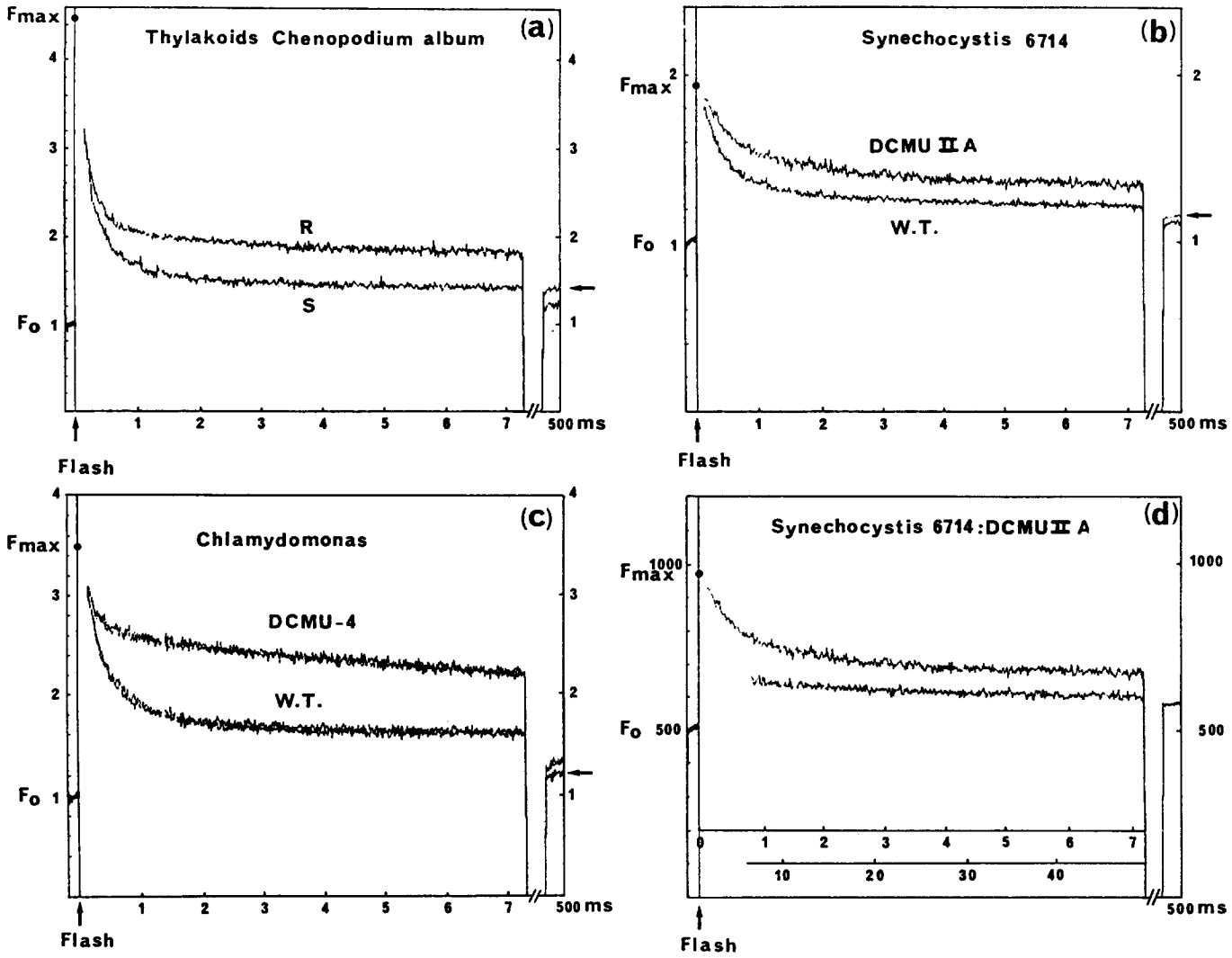

Fig. 1. Fluorescence decay after one saturating flash in dark adapted material. For detailed experimental conditions, see Materials and Methods. In a, b and c, the fluorescence of wild types and mutants is recorded between 120 μs and 7 ms. F_{max} is computed from curve fitting. The arrows on the right indicate in each case the F_{500ms} of the resistant mutant. In d, there are two recordings, one from 120 μs to 7 ms and a second from 7 to 40 ms.

some Q_A^- is not connected to the plastoquinone pool. In this case, it will react back with S_2 within seconds.

Fig. 1 shows fluorescence decay during the first milliseconds after a saturating flash for resistant and sensitive *Chenopodium album* thylakoids (a), for DCMU-IIA and wild type in *Synechocystis* 6714 (b) and for DCMU-4 and wild type in *C. reinhardtii* (c). The fluorescence level after 500 ms darkness was also measured. In all cases but one (DCMU-4 of *C. reinhardtii*), the fluorescence decayed within some milliseconds to a plateau.

The initial part of the decay was the same in all mutants as compared to wild type, but the quasi-stationary level was higher in the resistant than in the susceptible type and the difference remained at 500 ms after the flash. The fluorescence decay in the mutant DCMU-4 of *C. reinhardtii* was different. The fluorescence never leveled out to a plateau but it decayed steadily to a level close to that of wild-type strain at 0.5 s after the flash.

The fluorescence decays are polyphasic. The fluorescence intensity is not a linear indicator of Q_A^- concentration, therefore the phases resulting from a decomposition in a sum of exponentials are necessarily distorted. However, the decay during the first milliseconds after the flash with F_0 as the asymptotic level can be fitted in all cases by a sum of three exponentials. The half-time of the slower phase can be determined separately by a monoexponential fit of the decay between 10 and 40 ms after the flash (see Fig. 1d). It is then found to be in the time range of hundreds of milliseconds.

Within the precision of our measurements (the first point was taken at 120 μs after the flash), the half-time of the fast phase was between 200 and 400 μs, while that of the medium phase was between 1 and 3 ms.

Table II shows the corresponding amplitudes found for the three exponentials. The fluorescence decay analysis of IoxI and AzI mutants were very similar to the susceptible *Synechocystis.* In DCMU-IIA, DCMU-IIB and AzV of *Synechocystis,* in Taq 1 of *Synechococcus* and in resistant *C. album* chloroplasts, there was a significant increase of the slow phase amplitude with a concomitant decrease of the fast phase amplitude (around 40% of the total amplitude in DCMU-IIA and Taq 1 mutants).

In *C. reinhardtii* DCMU-4 mutant, the fluorescence decay was very different from that of the wild type, which corresponded to the general behavior described above. The second phase of the mutant was much slower ($t_{1/2} = 5$–7 ms). It is clear in Fig. 1c that the fluorescence level did not reach a plateau in the first 7 ms. The other difference between DCMU-4 mutant of *Chlamydomonas* and all the other mutants here studied was that, at 0.5 s after the flash, the fluorescence decayed back to a level close to that of the wild strain. Correspondingly, the miss parameter due to Q_A^- remaining at 0.5 s after the flashes is the same for the wild type and the DCMU-4 mutant of *Chlamydomonas* (cf. Table II).

Correlations between the slow phase of the fluorescence decay, the miss parameter and the thermoluminescence B band position

An increase in the miss parameter and a shift in the position of the thermoluminescence B band are all indicative of a displaced apparent equilibrium in oxygen-evolving centers.

There is a correlation between the amplitude of the fluorescence slow phase and both the miss parameter (Fig. 2) andn the B peak position (Fig. 3), for *Synechocystis* cells and *Chenopodium* chloroplasts. These data fit on a roughly linear regression.

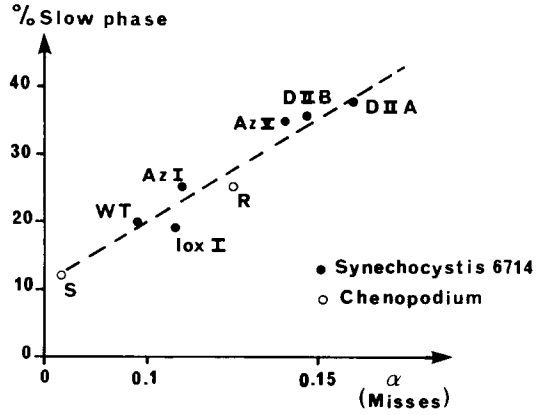

Fig. 2. Relationship between the amplitude of the slow phase of the fluorescence decline and the miss parameter computed from the oxygen oscillation pattern for the different mutants and wild type of *Synechocystis* 6714 and for R and S biotypes of *Chenopodium.*

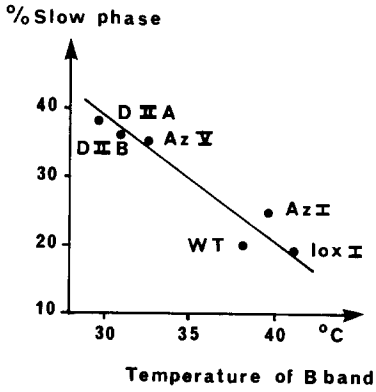

Fig. 3. Relationship between the amplitude of the fluorescence decline slow phase and the temperature of the B-band emission of thermoluminescence.

The correlation shows that the main effect of the mutations on the electron transfer is indeed an increase of Q_A^- concentration due to a change in the apparent equilibrium constant between Q_A^- and Q_B^-. In the case of *C. reinhardtii,* even the wild type was not on the regression line: the miss parameter (0.140) was much larger than in the wild type of *Synechocystis,* whereas the amplitudes of the fluorescence slow phases were equivalent.

Discussion

According to the results reported here, there are three types of mutation affecting electron transport of Photosystem II.

(i) The mutations with very small effects on the electron transfer: Phe-211 to Ser (AzI of *Synechocystis*), Asn-266 to Thr (IoxI of *Synechocystis*). These two mutants AzI and IoxI were weakly resistant to atrazine and ioxynil, respectively (cf. Table I). According to Erickson et al. [24], such unimpaired mutants were also found in *C. reinhardtii* and corresponded to substitutions Val-219 to Ile and Leu-275 to Phe.

(ii) The mutations which produced an appreciable change in electron transfer are the single mutation Ser-264 to Ala (DCMU-IIA of *Synechocystis*), Ser-264 to Gly (atrazine-resistant *C. album*) and double mutations in *Synechocystis* 6714, Ser-264 to Ala and Phe-255 to Leu (DCMU-IIB) and Phe-211 to Ser and Ala-251 to Val (AzV). However, the increased steady-state concentration of Q_A^- is not linked to a much slower Q_A^- reoxidation by Q_B (Fig. 1). We believe that the slow fluorescence decays previously reported for atrazine-resistant chloroplasts [15,16] could be in error because the sample was repeatedly illuminated using a 15 s dark time between flashes, leading to a partial reduction of the plastoquinone pool. Furthermore, the previous measurements were done at pH 7.8, where the stability of the resistant chloroplasts above 20 °C is poorer than

that of susceptible chloroplasts [25]. This does not occur at pH 6.5, the pH used in our measurements.

(iii) The mutation of Ser 264 to Ala in DCMU-4 of *C. reinhardtii* slows the reoxidation rate of Q_A^- by Q_B with no appreciable effect on the steady-state concentration of Q_A^-.

In cyanobacteria, transformation experiments have shown that the entire phenotype of the DCMU-IIA and AzV mutants could be ascribed to the D1 mutation [13,14]. In higher plants, such transformation experiments have not yet been possible and the field-collected mutants may have genetic variations in other traits in addition to that affecting the D1 protein.

The *psbA* gene is highly conserved in the four organisms. However, it is not identical in *Chlamydomonas,* higher plants and cyanobacteria. The *Chlamydomonas* D1 protein differs in the Q_B binding domain from D1 of cyanobacteria by several amino-acid residues and the same mutation of D1 does not produce the same modification in electron transfer and in herbicide resistance. A *Chlamydomonas* mutant with the Ser-264 changed to glycine would be most useful.

In conclusion, all examined substitutions of an amino-acid in the Q_B niche affect the electron transfer between Q_A and Q_B but to different extents. A comparison of various specific mutants of the same strain will help to further model Q_B and herbicide interaction with D1. It is noteworthy, however, that it may be harder to compare the effects of mutations of D1 in different organisms. Although the two-electron-gate mechanism is common to all Photosystem II centers, it is achieved with slightly different structures. Hence, the consequences of a particular mutation could depend on the D1 protein background.

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References

1 Tischer, W. and Strotmann, H. (1977) Biochim. Biophys. Acta 460, 113–125.
2 Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
3 Arntzen, C.J. and Doesing, J.H. (1984) in Advances in Gene Technology: Molecular Genetics of Plants and Animals (Ahmand, F. et al., eds.), Academic Press, New York.
4 Arntzen, C.J., Ditto, C.L. and Brewer, P.E. (1979) Proc. Natl. Acad. Sci. USA 76, 278–282.
5 Erickson, J., Rahire, M., Bennoun, P., Delepelaire, P., Diner, B. and Rochaix, J.D. (1984) Proc. Natl. Acad. Sci. USA 81, 3617–3621.
6 Astier, C., Vernotte, Der Vartanian, M. and Joset-Espardellier, F. (1979) Plant Cell Physiol. 20, 1501–1510.
7 Al Mouemar, A. (1983) Thesis, Université de Besançon.
8 Bettini, P., McNally, S., Sevignac, M., Darmency, H., Gasquez, J. and Dron, M. (1987) Plant Physiol. 84, 1442–1446.
9 Golden, S.S. and Haselkorn, R. (1985) Science 229, 1104–1107.
10 Ajlani, G., Meyer, I., Vernotte, C. and Astier, C. (1989) FEBS Lett. 246, 207–210.
11 Hirschberg, J. and McIntosh, L. (1983) Science 222, 1346–1349.
12 Goloubinoff, P., Edelman, M. and Hallick, R.B. (1984) Nuclear Acids Res. 12, 9489–9496.
13 Ajlani, G., Kirilovsky, D., Picaud, M. and Astier, C. (1989) Plant Mol. Biol., in press.
14 Kirilovsky, D., Ajlani, G., Picaud, M. and Etienne, A.L. (1989) Plant Mol. Biol., in press.
15 Pfister, K. and Arntzen, C.J. (1979) Z. Naturforsch. 34c, 996–1009.
16 Bowes, J., Crofts, A.R. and Arntzen, C.J. (1980) Arch. Biochem. Biophys. 200, 303–308.
17 Holt, J.S., Radosevich, S.R. and Stemler, A.J. (1983) Biochim. Biophys. Acta 722, 245–255.
18 Vermaas, W. (1984) Ph.D. thesis, Wageningen.
19 Demeter, S., Vass, I., Hideg, E. and Sallai, A. (1985) Biochim. Biophys. Acta 806, 16–24.
20 Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149–186.
21 Crofts, A.R., Robinson, H.H. and Snozzi, M. (1984) Advances in Photosynthesis Research, Vol. I, pp. 461–468, Martinus Nijhoff/Dr. W. Junk, Dordrecht.
22 Astier, C., Meyer, I., Vernotte, C. and Etienne, A.L. (1986) FEBS Lett. 207, 234–238.
23 Robinson, H., Golden, S., Brusslan, J. and Haselkorn, R. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 4, pp. 825–828.
24 Erickson, J.M., Pfister, K., Rahire, M., Togasaki, R.K., Mets, L. and Rochaix, J.D. (1989) Plant Cell 1, 361–371.
25 Ducruet, J.M. and Lemaine, Y. (1985) Plant Cell Physiol. 26, 419–429.
26 Boussac, A. and Etienne, A.L. (1982) Biochim. Biophys. Acta 682, 281–288.
27 Lemasson, C. and Etienne, A.L. (1975) Biochim. Biophys. Acta 408, 135–142.
28 Lavorel, J. (1978) J. Theor. Biol. 57, 171–185.
29 Bouges-Bocquet, B. (1973) Biochim. Biophys. Acta 325, 247
30 Rutherford, A.W., Crofts, A.R. and Inoue, Y. (1982) Biochim. Biophys. Acta 682, 457–465.
31 Demeter, S. and Vass, I. (1984) Biochim. Biophys. Acta 764, 24–32.
32 Wu, N.H., Coté, J.C. and Wu, R. (1987) Dev. Genet. 8, 339–350.