FEBS 21183 FEBS Letters 440 (1998) 59–63

Demonstration of thermal dissipation of absorbed quanta during energy-dependent quenching of chlorophyll fluorescence in photosynthetic membranes

Wafaa Yahyaoui, Johanne Harnois, Robert Carpentier*

Groupe de Recherche en Energie et Information Biomoléculaires, Université du Québec à Trois-Rivières, P.O. Box 500, Trois-Rivières, Que. G9A 5H7, Canada

Received 5 October 1998

Abstract When plant leaves or chloroplasts are exposed to illumination that exceeds their photosynthetic capacity, photoprotective mechanisms such as described by the energy-dependent (non-photochemical) quenching of chlorophyll fluorescence are involved. The protective action is attributed to an increased rate constant for thermal dissipation of absorbed quanta. We applied photoacoustic spectroscopy to monitor thermal dissipation in spinach thylakoid membranes together with simultaneous measurement of chlorophyll fluorescence in the presence of inhibitors of opposite action on the formation of ΔpH across the thylakoid membrane (tentoxin and nigericin/valinomycin). A linear relationship between the appearance of fluorescence quenching during formation of the ΔpH and the reciprocal variation of thermal dissipation was demonstrated. Dicyclohexylcarbodiimide, which is known to prevent protonation of the minor light-harvesting complexes of photosystem II, significantly reduced the formation of fluorescence quenching and the concurrent increase in thermal dissipation. However, the addition of exogenous ascorbate to activate the xanthophyll de-epoxidase increased non-photochemical fluorescence quenching without affecting the measured thermal dissipation. It is concluded that a portion of energy-dependent fluorescence quenching that is independent of de-epoxidase activity can be readily measured by photoacoustic spectroscopy as an increase in thermal deactivation processes.

Key words: Non-photochemical fluorescence quenching; Photoacoustic spectroscopy; Thylakoid membrane; Photosynthesis; Proton gradient; Xanthophyll

1. Introduction

Exposure of plant leaves or chloroplasts to illumination that exceeds their photosynthetic capacity leads to photoinhibition and degradation of the electron transport system. To avoid premature photoinhibition, excessive excitation energy can be dissipated by protective mechanisms such as described by the energy-dependent (non-photochemical) quenching of chlorophyll fluorescence [1,2]. It is thought that chlorophyll fluorescence is quenched by zeaxanthin (and antheraxanthin) molecules bound to the minor chlorophyll binding proteins of photosystem II [3,4]. Building of the pH gradient, presumably in the localized membrane domains [5], would activate the violaxanthin de-epoxidase with the resulting formation of antheraxanthin and zeaxanthin molecules [6] and promote the formation of binding sites on the chlorophyll binding proteins for these xanthophylls [7]. Protonation of the proteins would be a prerequisite for xanthophyll binding [4]. It is proposed that de-epoxidized xanthophylls could directly trap the singlet excited states of chlorophyll *a* whereas violaxanthin cannot [8]. Alternatively, it is suggested that the binding of zeaxanthin or protonation itself would favor an aggregated state of the chlorophyll binding proteins with the concurrent formation of weakly fluorescent traps for chlorophyll excited states [9]. Another hypothesis implies that the increased pH in the thylakoid lumen deactivates the oxygen evolving complex of photosystem II resulting in the formation of the oxidized primary donor P680$^+$ which would directly quench chlorophyll fluorescence [10].

The protective action of energy-dependent fluorescence quenching against excess light is attributed to an increased rate constant for radiationless energy dissipation processes in the antenna chlorophyll as calculated from chlorophyll fluorescence data [3]. Thermal dissipation processes can be measured by photoacoustic spectroscopy. It was recently shown, using this technique, that photochemical quenching of chlorophyll fluorescence due to open photosystem II reaction centers is directly correlated with a coinciding photochemical quenching of thermal dissipation [11–13]. An increased thermal dissipation during non-photochemical fluorescence quenching was also reported using photoacoustic spectroscopy [14–17], though some other reports concluded that energy-dependent quenching was not associated with significant modification in the yield of thermal dissipation [18,19].

In this report, we use photoacoustic spectroscopy to monitor thermal dissipation simultaneously with chlorophyll fluorescence in spinach thylakoid membranes under conditions where energy-dependent fluorescence quenching is observed. Various inhibitors were used to support the clear demonstration that energy-dependent fluorescence quenching is directly correlated with an increase in thermal deactivation processes.

2. Materials and methods

Thylakoid membranes were isolated from deveined spinach leaves as reported previously [20] and resuspended at 2 mg chlorophyll/ml in 330 mM sorbitol, 50 mM TES-NaOH (pH 7.5) and 2 mM $MgCl_2$. When required, the inhibitors were prepared in 80% acetone and less than 1% of the stock solution was mixed with the thylakoid preparations. Thylakoid membranes (250 μg chlorophyll/ml, 1 ml) were aspirated onto a nitrocellulose filter (Millipore, 0.4 μM pore size) using a gentle vacuum to obtain a homogeneous layer of photosynthetic membranes [21]. Simultaneous fluorescence and thermal dissipation measurements were performed with a laboratory-constructed system using a photoacoustic cell (MTEC Photoacoustic Inc., Ames, IA) in combination with a PAM-101 chlorophyll fluorometer (Walz, Effeltrich, Germany), as previously described [11,13].

---

*Corresponding author. Fax: (1) (819) 3765057.

PII: S0014-5793(98)01430-6

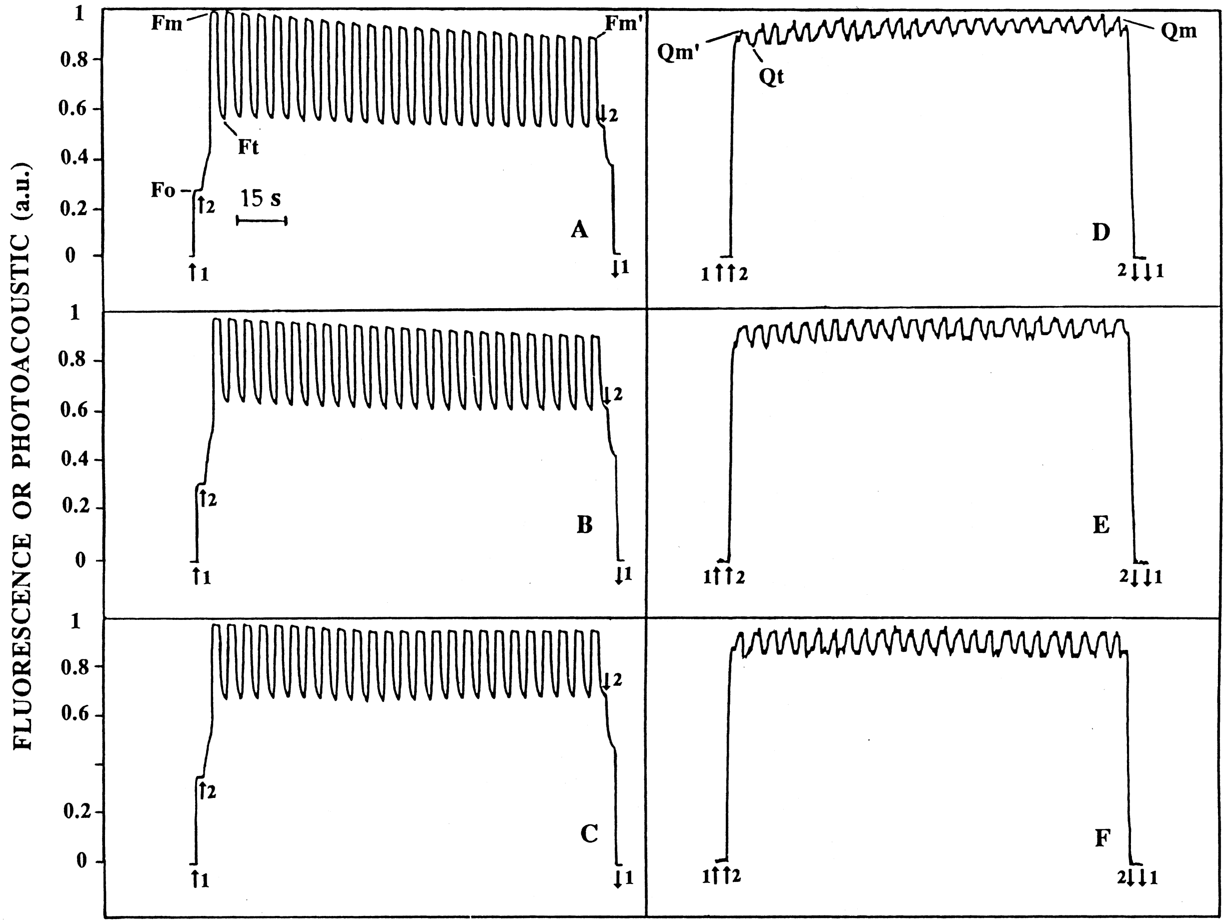

Fig. 1. Simultaneous recordings of fluorescence (left panels) and thermal dissipation (right panels) in thylakoid membranes. A and D: With the addition of 0.1 μM tentoxine. B and E: Control. C and F: With the addition of 5 μM nigericin and valinomycin. Up and down arrows indicate light on and off, respectively: light 1, weak 1.6 kHz fluorometer probe beam; light 2, intensity-modulated photoacoustic measuring beam (680 nm, 3.8 W/m^2, 35 Hz). Pulses of saturating white light are applied for 2 s each at 5 s intervals.

3. Results and discussion

Typical traces obtained from simultaneous recordings of chlorophyll fluorescence (pulse amplitude modulated fluorometer) and thermal dissipation (photoacoustic signal) in spinach thylakoid membranes are presented in Fig. 1. In these measurements, the weak 1.6 kHz fluorometer probe beam induces the fluorescence rise to the initial level F_o. The analogous thermal dissipation level Q_o is not detectable directly as explained in a previous report [11]. The photoacoustic signal Q_t is induced by an intensity-modulated measuring beam (680 nm, 3.8 W/m^2, 35 Hz). Upon absorption of this modulated light, thermal deactivation processes produce a pressure wave at the surface of the sample which is detected by a sensitive microphone connected to a lock-in amplifier. The modulated photoacoustic measuring beam also raises the fluorescence to an intermediate level of variable fluorescence F_t due to electron transport in photosystem II.

The total closure of the reaction centers is obtained by the further addition of saturating pulses of non-modulated light. This will provide the conditions for the specific measurement of non-photochemical quenching in photosystems where the photochemical activity is saturated. The 2 s pulse duration is required to provide an accurate acoustic signal measurement. The relatively weak intensity of the photoacoustic measuring beam and the low duration of saturating pulses together with the very short duration of an entire experiment (128 s) prevent any photoinhibition. The first pulse provides the maximal fluorescence level F_m and a thermal dissipation level Q_m'. During illumination with the photoacoustic measuring beam and the saturating pulses, a pH gradient is progressively formed across the thylakoid membranes and the energy-dependent fluorescence quenching gradually increases. The above causes the progressive decline of F_m to various F_m' levels. It is shown in Fig. 1 that this decline in chlorophyll fluorescence coincides with a gradual increase of the thermal dissipation Q_m' to the level Q_m that determines the maximal thermal dissipation obtained at the last pulse used.

Various terminology and equations have been used to quantify non-photochemical fluorescence quenching (NPQ) or the quenching coefficient q_N [22]. However, the increasing thermal dissipation from Q_m' to Q_m observed during the formation of the pH gradient across the thylakoid membrane (Fig. 1) is better quantified as $(Q_m-Q_m')/Q_m\times100\%$, a parameter that can be viewed as a coefficient of non-photochemical quenching of thermal dissipation, q_NQ. To use a term analogous to what can be used for thermal dissipation, non-photochemical fluorescence quenching will be expressed as $q_NF=(F_m-F_m')/F_m\times100\%$.

The values obtained at each saturating pulse for both q_NQ

and $q_N F$ are quantified in Fig. 2 (circles) together with the values obtained in the presence of two inhibitors of opposite action tentoxin and nigericin/valinomycin. Fig. 2 shows that $q_N F$ obtained from a control experiment such as shown in Fig. 1 increases with illumination period. Also, during the increase of $q_N F$, $q_N Q$ is progressively released (Fig. 2) as calculated from the increasing Q_m' values seen in Fig. 1. The quenching values are kept relatively small due to the short duration of the saturating illumination as mentioned above. Tentoxin, an uncompetitive inhibitor of the chloroplast CF1-ATPase [6], is known to induce an increase in ΔpH [23] and consequently an increase in energy-dependent fluorescence quenching, in comparison with the control experiments, is found in Fig. 2. Conversely, the couple nigericin/valinomycin, which is known to dissipate the electrochemical H^+ gradient, strongly reduced the fluorescence quenching. The corresponding variations of $q_N Q$ are also seen in Fig. 2. The thermal deactivation processes increased to an higher extent ($q_N Q$ decreases) in the presence of tentoxin in comparison with the control experiment which is apparent from a higher initial value of $q_N Q$. Further, the couple nigericin/valinomycin reduced the gradual increase in thermal deactivation coinciding with a weaker initial value of $q_N Q$. In these experiments, the inhibitors elicited only a partial effect in comparison with the expected action of these compounds at the concentrations used. However, the samples are aspirated onto a nitrocellulose filter before measurements. This procedure dramatically increases the chlorophyll/inhibitor ratio.

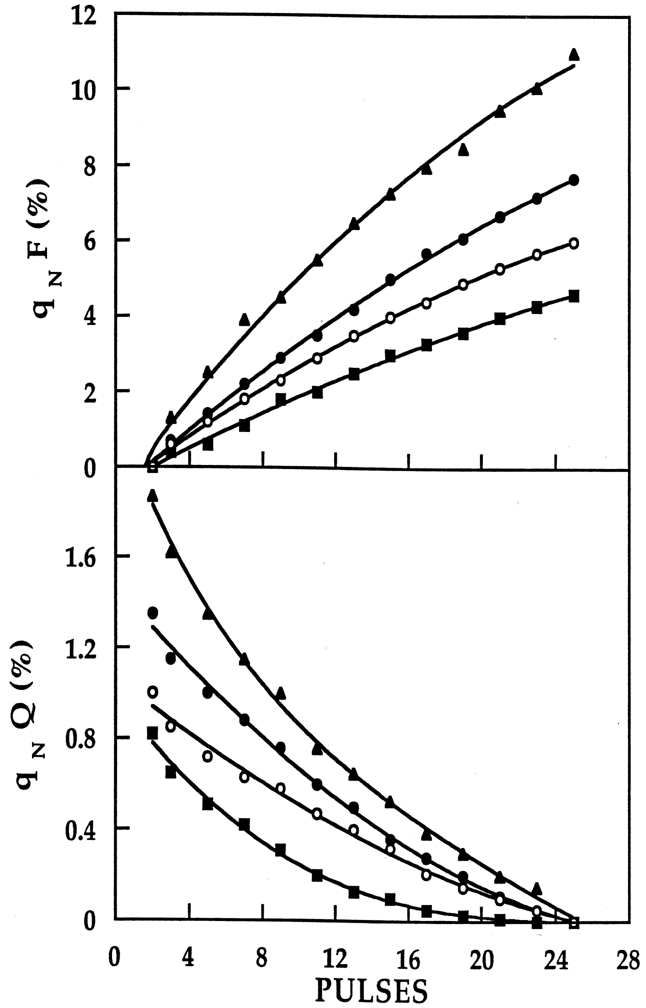

Fig. 2. Values for $q_N F$ and $q_N Q$ obtained from experiments such as presented in Fig. 1 as a function of the saturating pulse number: ●, without additive; ▲, with 0.1 μM tentoxin; ■, with 5 μM nigericin and valinomycin; ○, with 0.5 mM dicyclohexylcarbodiimide. The values for F_m' and Q_m' are obtained from averaging the values measured during a given saturating pulse.

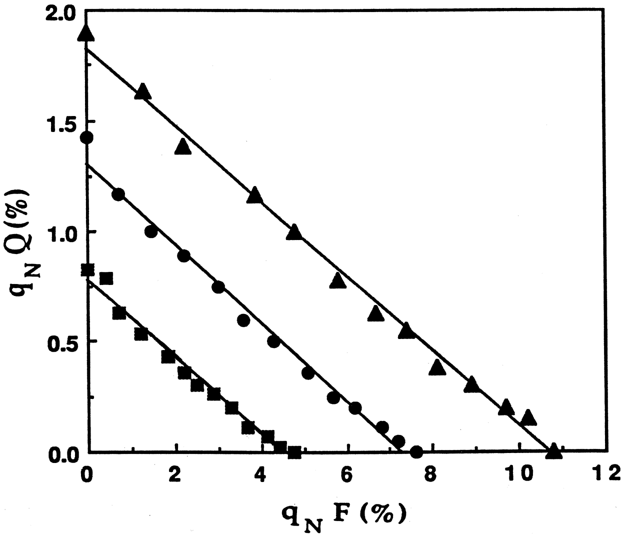

Fig. 3. Linear correlation between $q_N F$ and $q_N Q$ (the correlation factor R is given in parentheses) obtained from the data of Fig. 2: ●, without additive ($R = 0.988$); ▲, with 0.1 μM tentoxin ($R = 0.994$); ■, with 5 μM nigericin and valinomycin ($R = 0.980$).

In control experiments, an increase of $q_N F$ by 7.5% corresponded to a decrease of $q_N Q$ by 1.3% indicating that total fluorescence emission with the photosystems in the closed state accounted for about 14% of the de-excitations processes which nearly corresponds to the value of 10% previously suggested [24]. The values of $q_N Q$ and $q_N F$ were affected in similar proportion by the uncouplers (about 40% increase or decrease of maximal values, respectively, at the uncoupler concentrations used). Further, in all cases studied in Fig. 2, a clear linear relationship, with correlation factors near unity, was found between $q_N F$ and $q_N Q$ (Fig. 3). The above demonstrates that the changes in fluorescence intensity are strictly correlated with the increase in thermal dissipation and therefore that the energy-dependent fluorescence quenching is associated with a proportional increase in thermal deactivation processes.

As mentioned, protonation of pigment binding proteins may be a prerequisite for non-photochemical quenching of chlorophyll fluorescence. In line with the above, we have used the known inhibitor of energy-dependent fluorescence quenching dicyclohexylcarbodiimide (DCCD). It was shown that DCCD short circuits the proton pumping activity of photosystem II due to its binding to the minor pigment binding proteins and thus prevents protonation at these sites [25]. Interestingly, the inhibition of $q_N F$ by DCCD is accompanied by the corresponding effect on $q_N Q$ demonstrating that protonation of minor pigment binding proteins is involved in the increased thermal dissipation observed during fluorescence quenching.

It is demonstrated that activation of the enzyme violaxanthin de-epoxidase requires both low pH and ascorbate [26]. In isolated thylakoid preparations, endogenous ascorbate is re-

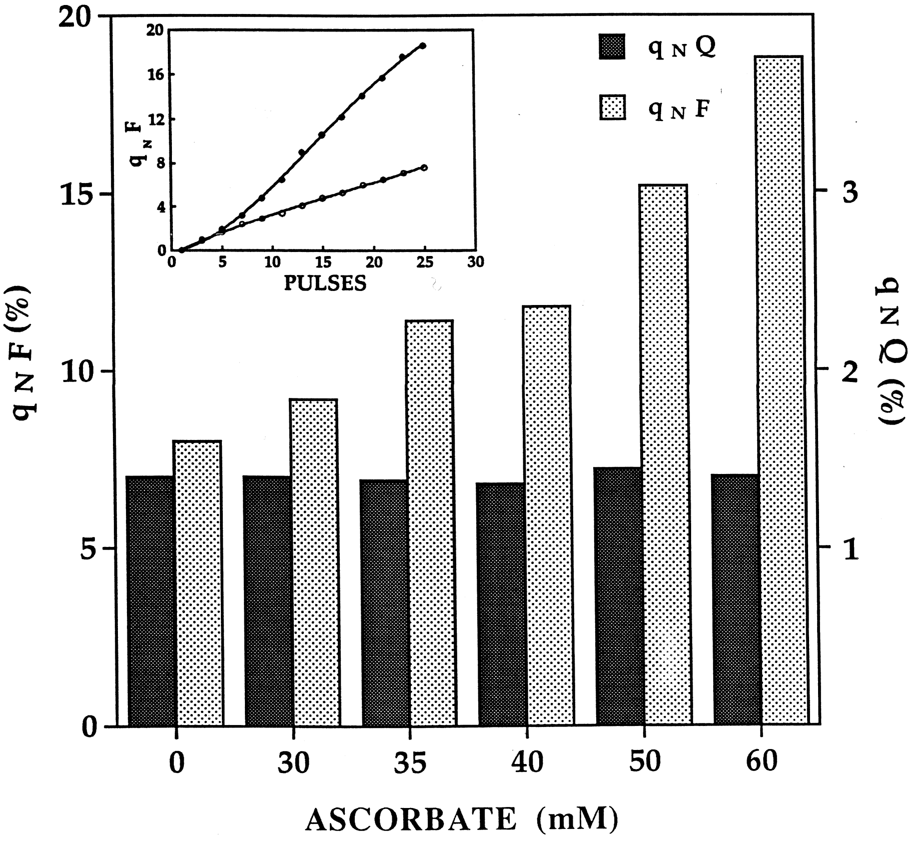

Fig. 4. Maximal values of $q_N F$ and $q_N Q$ obtained from data such as presented in Fig. 2 but with various ascorbate concentrations. The maximal values were obtained at the last pulse applied (25th) for $q_N F$ and at the first pulse for $q_N Q$. Inset: Values for $q_N F$ obtained from experiments such as presented in Fig. 1 as a function of the saturating pulse number: ○, without additive; ●, with 60 mM ascorbate. The values of F_m and Q_m were normalized to 1 before calculation.

moved during isolation procedures and the fluorescence quenching measured is likely independently from de-epoxidase activity. Ascorbate can be added to isolated thylakoid membranes or to the purified enzyme to accelerate the violaxanthin cycle [26]. As expected, addition of ascorbate to the thylakoid preparations resulted in a substantial increase in $q_N F$ (Fig. 4, inset) showing that the enhanced formation of de-epoxidized xanthophylls increased non-photochemical fluorescence quenching during illumination. This increase was dependent on ascorbate concentration (Fig. 4). Interestingly, $q_N Q$ remained totally unaffected by exogenous ascorbate at all the ascorbate concentrations used (Fig. 4).

The apparent absence of an increased thermal dissipation following the addition of ascorbate indicates that the enhanced thermal dissipation is not measured by the technique used. Indeed, only the events with a life-time short enough to follow the modulation frequency of the incident photoacoustic measuring beam are monitored by the corresponding pressure wave. It has been suggested that energy dissipation through a retarded pathway such as energy transfer from chlorophylls to carotenoids and thermal dissipation of the carotenoid triplet states may be responsible for a sufficient delay that would impair the detection by the modulated technique [27]. This is consistent with the lack of correlation between the increased thermal dissipation measured by photoacoustic spectroscopy and zeaxanthin formation during non-photochemical fluorescence quenching in leaves [16].

The present demonstration of the enhanced thermal dissipation of absorbed quanta in relation to the energy-dependent fluorescence quenching together with the modulation-independent positive action of exogenous ascorbate in the non-photochemical quenching coincides with the current mechanistic view of this photoprotection mechanism regarding the involvement of two different processes, one that depends on the de-epoxidase activity and the other that is independent of it. In the former process, fluorescence quenching may occur in the pigment binding proteins protonated upon formation of the localized pH gradient. In that case, newly de-epoxidized xanthophyll molecules could readily bind to their site to quench the excited states of chlorophyll [2,7]. Alternatively, the fluorescence quenching may proceeds through de-epoxidase-independent processes. Then, protonation of the pigment binding proteins could provide the conformational changes required for the formation of dissipating centers as previously proposed [9] or inhibition of oxygen evolution and formation of dissipating photosystem II reaction centers may be involved [10]. All these de-epoxidase-independent processes are likely to proceed at the modulation frequency of the incident photoacoustic measuring beam because they are directly related to the formation of the ΔpH. Our results showing the inhibition of $q_N Q$ in the presence of DCCD demonstrate that the protonation of pigment binding proteins is implicated in the enhanced thermal dissipation. Those data favor the hypothesis of thermal dissipation in the pigment bead.

Acknowledgements: The authors thank A. Tessier for his professional assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

References

[1] Demming-Adams, B. (1990) Biochim. Biophys. Acta 1020, 1–24.
[2] Gilmore, A.M. (1997) Physiol. Plant. 99, 197–209.
[3] Walters, R.G., Ruban, A.V. and Horton, P. (1994) Eur. J. Biochem. 226, 1063–1069.
[4] Pesaresi, P., Sandonà, D., Giuffra, E. and Bassi, R. (1997) FEBS Lett. 402, 151–156.
[5] Pfündel, E.E., Renganathan, M., Gilmore, A.M., Yamamoto, H.Y. and Dilley, R.A. (1994) Plant Physiol. 106, 1647–1658.
[6] Hu, D., Fiedler, H.R., Golan, T., Edelman, M., Strotmann, H., Shavit, N. and Leu, S. (1997) J. Biol. Chem. 272, 5457–5463.
[7] Gilmore, A.M., Hazlett, T.L., Debrunner, P.G. and Govindjee (1996) Photochem. Photobiol. 64, 552–563.
[8] Frank, H.A., Cua, A., Chynwat, V., Young, A., Gosztola, D. and Wasielewski, M.R. (1994) Photosynth. Res. 41, 389–395.
[9] Ruban, A.V. and Horton, P. (1992) Biochim. Biophys. Acta 1102, 30–38.
[10] Bruce, D., Samson, G. and Carpenter, C. (1997) Biochemistry 36, 749–755.
[11] Allakhverdiev, S.I., Klimov, V.V. and Carpentier, R. (1994) Proc. Natl. Acad. Sci. USA 91, 281–285.
[12] Markovic, D. and Carpentier, R. (1995) Biochem. Cell Biol. 73, 247–252.
[13] Yahyaoui, W., Markovic, D. and Carpentier, R. (1997) Opt. Eng. 36, 337–342.
[14] Snel, J.F.H., Kooijman, M. and Vredenberg, W.J. (1990) Photosynth. Res. 25, 259–268.
[15] Dau, H. and Hansen, U. (1990) Photosynth. Res. 25, 269–278.
[16] Havaux, M. and Tardy, F. (1997) J. Photochem. Photobiol. B. 40, 68–75.
[17] Mullinaux, C.W., Ruban, A.V. and Horton, P. (1994) Biochim. Biophys. Acta 1185, 119–123.
[18] Buschmann, C. and Kocsanyi, L. (1989) Photosynth. Res. 21, 129–136.
[19] Havaux, M. (1990) Photochem. Photobiol. 51, 481–486.
[20] Purcell, M., Leroux, G.D. and Carpentier, R. (1990) Pestic. Biochem. Physiol. 37, 83–89.
[21] Velitchkova, M. and Carpentier, R. (1994) Photosynth. Res. 40, 263–268.
[22] Demmings-Adams, B., Adams III, W.W., Baker, D.H., Logan, B.A., Bowling, D.R. and Verhoeven, A.S. (1996) Physiol. Plant. 98, 253–264.
[23] Quick, P., Sheibe, R. and Stitt, A. (1989) Biochim. Biophys. Acta 974, 282–288.
[24] Lavorel, J. and Etienne, A.L. (1977) in: Primary Processes in Photosynthesis (Barber, J., Ed.), pp. 203–268, Elsevier/North Holland, Amsterdam.
[25] Jahns, P. and Junge, W. (1990) Eur. J. Biochem. 193, 731–736.
[26] Bratt, C.E., Arvidsson, P.-O., Carlsson, M. and Akerlund, H.-E. (1995) Photosynth. Res. 45, 169–175.
[27] Gruszecki, W.I., Wojtowicz, K., Krupa, Z. and Strzalka, K. (1994) J. Photochem. Photobiol. B. 22, 23–27.