Turnover of the D-1 Reaction Center Polypeptide from Photosystem II in Intact Spruce Needles and Spinach Leaves

Doris Godde

Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum,
Postfach 10 21 48, D-4630 Bochum 1, Bundesrepublik Deutschland

Heribert Schmitz and Manfred Weidner

Botanisches Institut, III. Lehrstuhl, Universität zu Köln,
Gyrhofstraße 15, D-5000 Köln, Bundesrepublik Deutschland

A new method is presented to follow the turnover of the D-1 reaction center polypeptide of photosystem II in intact spruce needles or spinach leaves by incorporation of [^{14}C]leucine. Like in other organisms the synthesis and degradation of this protein is strongly light-dependent and very fast. The synthesis rate under moderate light intensities in healthy spruce needles is about 9 nmol [^{14}C]leucine mg^{-1} Chl h^{-1}. The half-life time of the D-1 protein under these conditions is less than 30 min. In order to demonstrate the usefulness of this method for the stress physiology of photosynthesis, first experiments are shown comparing the turnover of the D-1 protein in spruce needles with different age and different degree of damage.

Introduction

Inhibition of photosynthesis by excess light intensities is a long-known phenomenon. It is primarily due to an inactivation of photosystem II (see review [1, 2]). In intact plants it is strongly related to the rapid turnover [3−5] of the D-1 reaction center polypeptide of photosystem II [5−7]. In the light, this protein is continuously damaged, degraded, removed from the membrane, newly synthesized and again inserted. When the rate of degradation exceeds the rate of synthesis, which is the case at excess light intensities, the content of the D-1 protein in the thylakoid membrane decreases. As a consequence, the other reaction center components are also degraded and photosystem II is finally destroyed [8].

Photoinhibition can be enhanced by stress factors, such as coldness, heat and drought (see review [9−11]). Under these conditions photoinhibitory damage is observed even at moderate light conditions. Thus, photoinhibition appears as a widespread phenomenon in the field under natural conditions [12] and is perhaps aggravated by anthropogenic environmental factors, *e.g.* air pollutants [11] and mineral deficiencies. It may be assumed that photoinhibitory processes also play an important role in damaged spruce trees suffering under the symptoms of novel forest decline.

It has been proposed by Kyle and Ohad [2, 13] that stressors change the susceptibility of a plant against photoinhibition by altering the equilibrium between the synthesis and the degradation rate of the D-1 protein. Most experiments investigating the turnover of the D-1 protein have been done with unicellular algae, with small water plants or cell organelles, which are not comparable to field plants [3, 4, 6, 7, 14, 15].

Here we wish to present a method which allows to study the turnover of the D-1 protein in field plants and trees like spinach and spruce by radioactively labelling this protein with [^{14}C]leucine. First results indicate that the synthesis of the D-1 protein in spruce depends on the needle age and differs with the degree of damage.

Methods

Branches of a severely damaged spruce tree (stand Velmerstot, Nordrhein-Westfalen, damage class 3) and a healthy tree (stand Glindfeld, Nordrhein-Westfalen, F.R.G., damage class 0) were harvested at May 25th and August 20th 1990, respectively, and were incubated with [^{14}C]leucine the following day. The classification of the trees was done by the Landesanstalt für Ökologie,

Landschaftsentwicklung und Forstplanung, Recklinghausen, F.R.G., as a contribution to the research program of the Ministerium für Umwelt, Raumordnung und Landwirtschaft, Nordrhein-Westfalen "Airpollution and Forest Decline".

Otherwise, branches of spruce trees (4 years old, grown in a cold-house) and spinach plants were freshly harvested. 0.7 g spruce needles were cut from the branches and immediately put into an evacuable glass vessel. Spinach leaves were first cut with a razor blade into small pieces of $0.25\ cm^2$. According to Weidner [16], 10 ml of a 1 mM leucine solution containing 9250 MBq [^{14}C]leucine were added under dim light and sucked into the plant material by vacuum infiltration. The vessel was put into a shaking apparatus in a water bath at a temperature of 25 °C. The leaf material was illuminated for different times with white light of 250 Wm^{-2}. After illumination it was instantly taken from the incubation medium and frozen in liquid nitrogen.

For the chase experiments, spruce needles were incubated in the light for 45 min in a solution containing 1 mM [^{14}C]leucine with a specific radioactivity of 9250 MBq. The needles were washed in the dark with water and immediately put in a solution containing 10 mM unlabelled leucine. After illumination for different times the needles were taken out and immediately frozen in liquid nitrogen.

After washing the plant material with distilled water, thylakoids from spinach leaves were prepared according to standard methods [17] using a Waring Blender equipped with a minibeaker MC-1. In order to prepare thylakoids from spruce, needles were homogenized for 2 min in a Waring Blender equipped with a minibeaker with 15 ml isolation medium containing 1 M sorbitol, 50 mM tricine/KOH, pH 7.5, 2 mM $MgCl_2$ and 1 mM $MnCl_2$. The homogenate was filtered through nylon gaze. After centrifugation at $3000 \times g$ for 5 min, the green pellet was washed once with isolation medium. After the second centrifugation the chloroplasts were broken up with distilled water and centrifuged at $5000 \times g$.

Chlorophyll was determined according to Arnon [18].

The relative content per chlorophyll of the D-1 protein was determined immunologically by Western blotting. The antibody against the D-1 protein was a generous gift by Prof. R. Berzborn (Ruhr-Universität, Bochum). Spinach or spruce thylakoids were first dissolved in 5% SDS, 15% glycerol, 50 mM Tris, pH 6.8 and 2% mercaptoethanol at room temperature and, if necessary sonicated with the microtip of a Branson sonifier for 2 times 15 sec. The polypeptides were separated by polyacrylamide gel (15%) electrophoresis according to Schägger *et al.* [19].

The polypeptides were then transferred to nitrocellulose by electroblotting in a BioRad Transblot chamber for 3 h at 0.4 A at -28 °C. After saturation with 3% gelantine in Tris buffer, pH 7.5 the first antibody was allowed to react overnight at room temperature in 1% gelantine. After washing with Tris and 0.05% Tween-20 the second antibody (horseradish peroxidase-conjugated) was allowed to react in 1% gelantine for 2 h, and developed with HPR colour development (BioRad) and 0.005% H_2O_2.

SDS gels or Western blots were exposed to Hyperfilm-MP (Amersham-Buchler) for 2 to 4 days. The densitometry of the autoradiograms was performed with a LKB Ultroscan XL Laser Densitometer.

Results

Photoinhibition *in vivo* is strongly related to the rapid turnover of the D-1 reaction center polypeptide [3–5] of photosystem II [5–7]. So far, this turnover could only be measured in unicellular algae, small water plants or in intact chloroplasts of higher plants [3, 4, 6, 7, 14, 15]. Since photoinhibition is a common phenomenon also observed in the field [11, 12], it was desirable to study the synthesis and degradation of this protein also in field plants. Here we wish to present a method which allows to measure the turnover of the D-1 protein in leaves of higher plants such as spruce or spinach.

It is known from investigations of the total protein synthesis that [^{14}C]leucine is easily taken up by plant material and is therefore well suited to study the turnover of proteins with a short half-life time [15, 20, 21]. As can be seen from Fig. 1, spruce needles easily incorporate [^{14}C]leucine into their thylakoidal proteins. We found an average incorporation rate of 3.77 kBq [^{14}C]leucine mg^{-1} Chl h^{-1} or 40 nmol leucine mg^{-1} Chl h^{-1}. The autoradiogram shows that this incorporation is complete-

Western-Blot   Autoradiogram

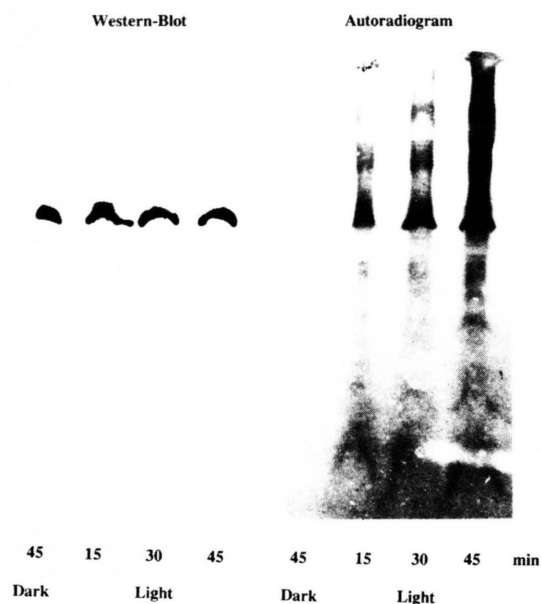| 45 | 15 | 30 | 45 | 45 | 15 | 30 | 45 | min |
| Dark | | Light | | Dark | | Light | | |

Fig. 1. Incorporation of [^{14}C]leucine into the D-1 protein of spruce thylakoids as identified by Western blotting. 0.7 g spruce needles from the first needle year of a 4 year old tree were incubated in a 1 mM leucine solution containing 9250 MBq [^{14}C]leucine for different times. Isolation of thylakoids, SDS gel electrophoresis and Western blotting with an antibody against the D-1 protein were carried out as described in Materials and Methods.

Fig. 2. Time Dependence of the incorporation of [^{14}C]leucine into the thylakoid proteins of spruce. The incorporation of [^{14}C]leucine was carried out as described in Materials and Methods.

ly light-dependent. Almost no radioactivity could be detected, if the needles were incubated in the dark. But already after 15 min illumination with moderate light intensities one prominent protein band was radioactively labelled. The fast light-dependent turnover is characteristic of the D-1 reaction center protein of photosystem II [4]. By the method of Western blotting the labelled polypeptide could indeed be identified as the D-1 reaction center polypeptide of photosystem II (Fig. 1). The incorporation of [^{14}C]leucine into the D-1 protein of spruce is linear up to 45 min (Fig. 2).

To estimate the radioactivity incorporated into the D-1 protein, the density of the autoradiogram was measured with a laser scanner. More than 30% of the radioactivity was found in this single protein (Fig. 3). This means that 12 nmol leucine are incorporated in 1 h into the D-1 protein per mg chlorophyll. A second area of high radioactivity can be found at the starting position of the SDS gel
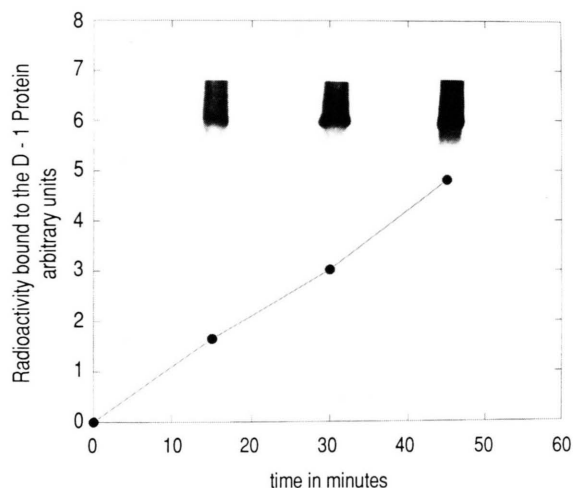
and might be due to unsolubilized material. We also determined the incorporation of [^{14}C]leucine into the D-1 protein by cutting and solubilizing the SDS gel. The rate of incorporation calculated from these data is 9 nmol [^{14}C]leucine mg^{-1} Chl h^{-1}.

Like its synthesis also the degradation of the D-1 protein is known to be very fast and strongly light-dependent [4]. This is also true for the D-1 protein from spruce as can be seen from the chase experiment shown in Fig. 4. At first the D-1 protein was radioactively labelled in the light. No incorporation of [^{14}C]leucine was observed in the dark. After 15 min chase period in the light with a
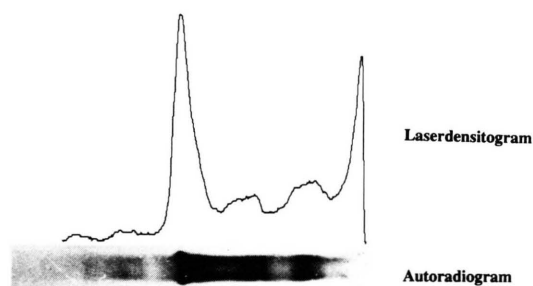

Fig. 3. Laser densitogram of an autoradiogram of thylakoidal proteins labelled with [^{14}C]leucine. The autoradiogram of labelled thylakoidal proteins from spruce was taken as described in Materials and Methods.
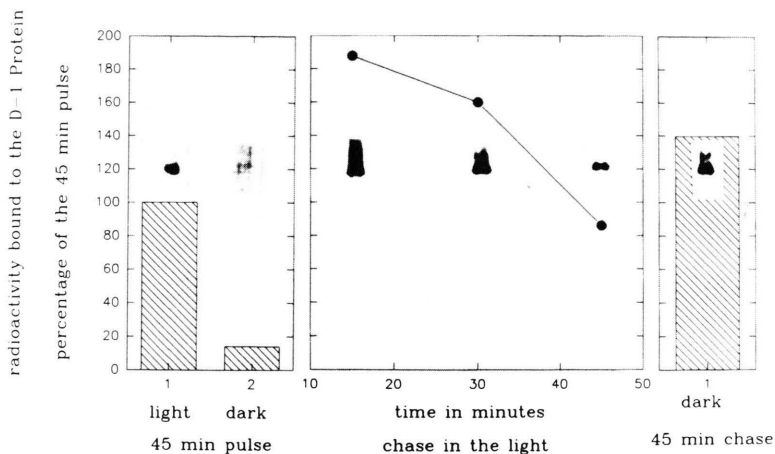
Fig. 4. Light-dependent degradation of the D-1 protein of spruce thylakoids. The pulse-chase experiment was carried out with spruce needles of the new flush as described in Materials and Methods.

tenfold higher concentration of unlabelled leucine the radioactivity incorporated into the D-1 protein increased up to 180% of the value measured after the 45 min pulse. This was probably due to the big pool size of leucine. In the next 30 min the radioactivity dropped to 70% of the original activity. The half-life time of the D-1 protein calculated from these data is 27 min. After 45 min chase in the dark the radioactivity incorporated into the D-1 protein compared to the pulse after 45 min in the light has increased from 100% to 140%. This is probably due to residual amounts of mRNA having been synthesized in the preceding light phase. This indicates that no degradation of the D-1 protein has taken place in the dark.

The method presented is also suitable to follow the turnover of the D-1 protein in intact spinach leaves. Fig. 5 shows the autoradiogram of spinach thylakoid proteins labelled with [14C]leucine in the dark and in the light. Again the radioactive amino acid was incorporated into one major protein, which could be identified by Western blotting as the D-1 protein. Like in spruce, the synthesis of the D-1 protein is completely light-dependent. Only small amounts of radioactivity could be detected in the thylakoid proteins from spinach leaves incubated in the dark.

According to the hypothesis of Kyle and Ohad [2, 13] stress conditions should alter the turnover of the D-1 protein, thus changing the sensitivity of plants against photoinhibition. To demonstrate that indeed the turnover of the D-1 protein does not only depend on the light regime but also on other external and internal conditions of a plant,

the synthesis rate of the D-1 protein was followed over the year in needles of two healthy and severely damaged spruce trees, exhibiting chlorosis and needle loss. Figure 6 shows the incorporation of [14C]leucine into the D-1 protein of these trees. The experiment was carried out in August, when the needles were in the same developmental stage. As can be seen from the Western blot, the content of the D-1 protein related to chlorophyll is higher in the needles of the healthy trees as compared to the damaged ones. From the autoradiogram it can be
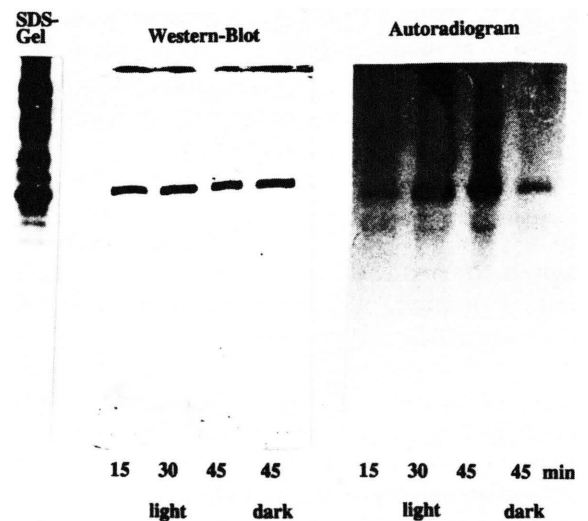

Fig. 5. Incorporation of [14C]leucine into the D-1 protein of spinach leaves. 2 g spinach leaves were incubated with [14C]leucine as described in Materials and Methods.
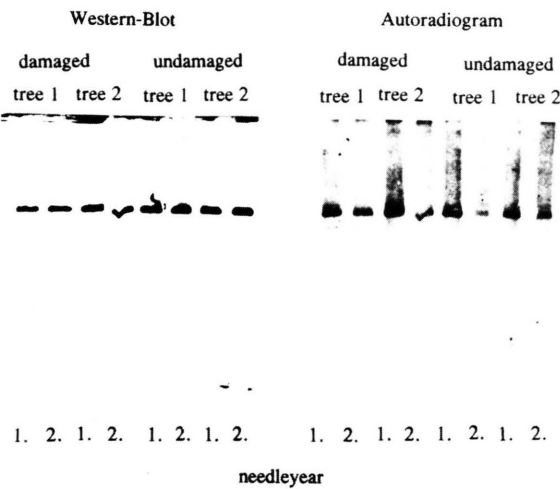
Fig. 6. Synthesis of the D-1 protein in damaged and non-damaged spruce (harvested August 1990). The incorporation of [^{14}C]leucine into the spruce thylakoid proteins was measured as described in Materials and Methods. The D-1 protein was identified by Western blotting.

Fig. 7. Synthesis of the D-1 protein in damaged and non-damaged spruce (harvested May 1990). The incorporation of [^{14}C]leucine into spruce thylakoid proteins was measured as described in Materials and Methods. The D-1 protein was identified by Western blotting.
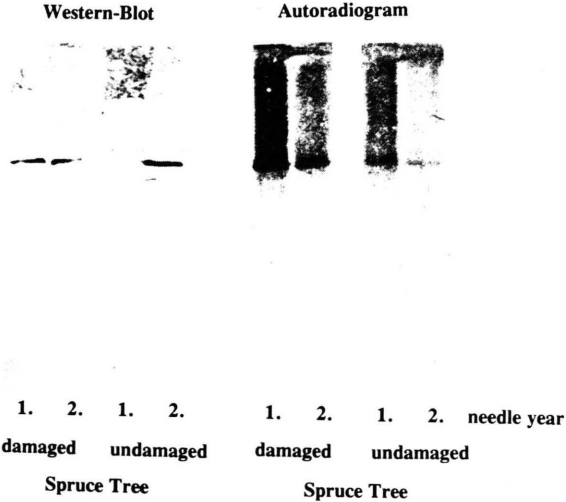
seen, however, that the synthesis of the D-1 protein is generally higher in the damaged trees than in the undamaged trees. The lowest rate of the synthesis of the D-1 protein was found in the second needle year of the undamaged trees, although these needles were found to have the highest content of the D-1 protein. Such differences in the turnover of the D-1 protein in damaged and undamaged trees were found all over the vegetation period.

The greatest differences in the synthesis of the D-1 protein could be seen during the development of the new flush (Fig. 7), a time, when trees are known to react very sensitively towards stress conditions [22]. Again the synthesis of the D-1 protein is generally higher in the damaged tree than in the undamaged tree regardless of the needle age. However, the synthesis of the D-1 protein also depends on the age of the needles. In the developing flush the synthesis of the D-1 protein is generally faster in the one year old needles regardless of the degree of damage. This indicates that the turnover of the D-1 protein in a leaf is not only determined by the light intensities. Also other factors like leaf development and age, internal and external stress factors seem to alter the turnover of the D-1 protein of photosystem II as well.

Discussion

The D-1 protein is one of the reaction center proteins of photosystem II [23–25]. It is characterized by a rapid turnover, which is completely light-dependent [3–5]. Its synthesis takes place as membrane-bound precursor within the chloroplast, where it is integrated first into the stromal lamellae. Then in a very fast reaction of several minutes, the processed form is transferred to the granal lamellae [26]. We were able to follow the rapid turnover of the D-1 protein in intact spruce needles and spinach leaves by incorporation of [^{14}C]leucine into the thylakoid protein. Like in other organisms, the synthesis of the D-1 protein is under complete light control and very fast. In spruce needles the incorporation rate of [^{14}C]leucine was found to be 9 nmol leucine mg Chl^{-1} h^{-1}. More than 30% of the radioactivity bound to the thylakoid proteins was incorporated into the D-1 protein.

Like the synthesis of the D-1 protein also its degradation is light-dependent and very fast [4]. According to Edelman [27] this light-dependent rapid turnover of the D-1 protein is an adaptation to oxygenic photosynthesis. The primary cleavage of the D-1 protein occurs between arg 238 and ile 248, directly after a PEST-like sequence [27,

28]. The D-1 protein is the only thylakoidal protein with a PEST-like sequence. Interestingly, the homologous L subunit of the anoxygenic bacterial reaction center which is stable in the light has no PEST sequence.

In *Dunaliella* [15] and *Spirodela* [28] the half-life time is in the range of several hours. For spruce we found a half-life time of about 30 min. This short half-life time might be due to differences in the light regime. But also the submerse incubation of leaves which is quite unnatural might be an additional stressor which increases the turnover of the D-1 protein.

The rapid turnover of the D-1 reaction center protein is related to photoinhibition [5−7]: the D-1 protein is damaged by excess light, then degraded and replaced by a newly synthesized protein. When the degradation rate outbalances the synthesis, photosystem II cannot be repaired and is finally degraded [8]. The exact mechanism of this damage is not well understood. It has been proposed that the initial stage of photoinactivation is located at the electron donor site of photosystem II [29]. But also mutations of the D-1 protein at the acceptor site which alter the semiquinone binding change the rapid turnover and result in a faster photoinhibition [30].

It has been proposed that stress factors could alter the turnover of the D-1 protein thus changing the susceptibility of a plant against photoinhibitory damages [2, 13]. First results of Lütz and Godde [31] have shown that indeed in fumigated spruce trees the content of the D-1 protein is readily influenced by stress factors like air pollutants. At low concentrations of air pollutants it first increases, whereas at high load the content of the D-1 protein finally decreases. This indicates that stressors stimulate the turnover of this protein.

In this context, we looked at the synthesis rates of D-1 protein in needles from spruce trees which differ in their degree of damage. We found that the turnover of the D-1 protein does not only depend on the light regime, but also on other internal and external factors. The synthesis of this protein is generally higher in the new flush than in second year needles, probably due to the fact that the whole photosynthetic apparatus must be synthesized *de novo* in the newly developing needles. This difference is very much pronounced in spring, but continues over the whole vegetation period. Obviously, the photosynthetic apparatus is not so stable in the young needles as compared to the older ones. In the damaged trees the synthesis of the D-1 protein is generally increased when compared to the undamaged trees, regardless of needle age and vegetation period. However, the content of the D-1 protein was often found to be reduced in the damaged trees, especially in the second year needles. Thus, the turnover of the D-1 protein is supposedly higher in the damaged needles than in the undamaged needles. This might indicate, that indeed stress factors could alter the turnover of the D-1 protein. Whether this has any impact with regard to the understanding of modern forest decline has to be proved by further experiments both with trees which are exposed to defined stress conditions and with trees from forest stands.

[1] S. B. Powles, Annu. Rev. Plant Physiol. **35**, 15–44 (1984).
[2] D. J. Kyle, in: Photoinhibition (D. J. Kyle, C. B. Osmond, and C. J. Arntzen, eds.), Topics in Photosynthesis, **Vol. 9**, pp. 197–222 (J. Barber, ed.), Elsevier, Amsterdam 1987.
[3] H. Hoffmann-Falk, A. K. Mattoo, J. B. Marder, M. Edelmann, and R. J. Ellis, J. Biol. Chem. **257**, 4583–4587 (1982).
[4] A. K. Mattoo, H. Hoffmann-Falk, J. B. Marder, and M. Edelmann, Proc. Natl. Acad. Sci. U.S.A. **81**, 1380–1384 (1984).
[5] I. Ohad, D. J. Kyle, and J. Hirschberg, EMBO J. **4**, 1655–1659 (1985).
[6] D. J. Kyle, I. Ohad, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **81**, 4070–4074 (1984).
[7] I. Ohad, D. J. Kyle, and C. J. Arntzen, J. Cell Biol. **99**, 481–485 (1984).
[8] G. Schuster, R. Timberg, and I. Ohad, Eur. J. Biochem. **177**, 403–410 (1988).
[9] G. Öquist, D. H. Greer, and E. Ögren, in: Photoinhibition (D. J. Kyle, C. B. Osmond, and C. J. Arntzen, eds.), Topics in Photosynthesis, **Vol. 9**, pp. 67–88 (J. Barber, ed.), Elsevier, Amsterdam 1987.
[10] M. M. Ludlow, in: Photoinhibition (D. J. Kyle, C. B. Osmond, and C. J. Arntzen, eds.), Topics in Photosynthesis, **Vol. 9**, pp. 89–110 (J. Barber, ed.), Elsevier, Amsterdam 1987.
[11] G. H. Krause, Physiol. Plant. **74**, 566–574 (1988).
[12] E. Ögren and M. Sjöström, Planta **181**, 560–567 (1990).
[13] D. J. Kyle and I. Ohad, in: Photosynthesis III. Photosynthetic Membranes (L. A. Staehelin and C. J. Arntzen, eds.), Encyclopedia of Plant Physiology, New Series, **Vol. 19**, pp. 468–472, Springer Verlag, Berlin 1986.
[14] H. Gong and S. Nilsen, J. Plant Physiol. **135**, 9–14 (1989).
[15] K. Gounaris, U. Pick, and J. Barber, FEBS Lett. **211**, 94–98 (1987).
[16] M. Weidner, B. Engel, M. Kraus, U. Löffel, and B. Schmidt, Forschungsberichte zum Forschungsprogramm des Landes Nordrhein-Westfalen "Luftverunreinigungen und Waldschäden" Nr. 4: "Die Assimilation von Kohlenstoff und Stickstoff unter dem Einfluß von Luftschadstoffen. Enzymologische und transportphysiologische Untersuchungen. Teil 2" (Der Minister für Umwelt, Raumordnung und Landwirtschaft des Landes Nordrhein-Westfalen, ed.), Thierbach, Mühlheim 1989, ISSN 0934-5124.
[17] N. Nelson, Z. Drechsler, and J. Neumann, J. Biol. Chem. **245**, 143–151 (1970).
[18] D. I. Arnon, Plant Physiol. **24**, 1–25 (1949).
[19] H. Schägger, U. Borchert, H. Aquila, T. A. Link, and G. v. Jagow, FEBS Lett. **190**, 89–94 (1985).
[20] M. Weidner and C. Ziemens, Plant Physiol. **56**, 590–594 (1976).
[21] M. Weidner and G. Combrink, Plant Physiol. **64**, 144–149 (1976).
[22] O. L. Lange, R. M. Weikert, M. Wedler, J. Gebel, and U. Heber, AFZ **3**, 55–64 (1989).
[23] A. Trebst, Z. Naturforsch. **41c**, 240–245 (1985).
[24] O. Naba, K. Satoh, Proc. Natl. Acad. Sci. U.S.A. **84**, 109–112 (1987).
[25] J. Barber and J. B. Marder, in: Biotechnology and Genetic Engineering Reviews (G. E. Russel, ed.), pp. 355–404, Intercept, Newcastle-upon-tyne 1986.
[26] A. K. Mattoo and M. Edelman, Proc. Natl. Acad. Sci. U.S.A. **84**, 1497–1501 (1987).
[27] A. K. Mattoo, J. B. Marder, and M. Edelman, Cell **56**, 241–246 (1987).
[28] B. M. Greenberg, V. Gaba, A. K. Mattoo, and M. Edelman, EMBO J. **6**, 2865 (1987).
[29] D. J. Blubaugh and G. M. Cheniae, Biochemistry **29**, 5109–5118 (1990).
[30] N. Ohad, D. Amir-Shapira, H. Koike, Y. Inoue, I. Ohad, and J. Hirschberg, Z. Naturforsch. **45c**, 402–408 (1990).
[31] C. Lütz and D. Godde, Physiol. Plant. **79**, A 125 (1990).