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Photoinhibition and light-dependent turnover of the D1 reaction-centre polypeptide of photosystem II are enhanced by mineral-stress conditions

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Abstract. The function of photosystem II (PSII) and the turnover of its D1 reaction-center protein were studied in spinach (*Spinacia oleracea* L.) plants set under mineral stress. The mineral deficiencies were induced either by supplying the plants with an acidic nutrient solution or by strongly reducing the supply of magnesium alone or together with sulfur. After exposure for 8–10 weeks to the different media, the plants were characterized by a loss of chlorophyll and an increase in starch content, indicating a disturbance in the allocation of assimilates. Depending on the severity of the mineral deficiencies the plants lost their ability to adapt even to moderate iradiances of 400 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and became photoinhibited, as indicated by the decrease in F_v/F_m (the ratio of yield of variable fluorescence to yield of maximal fluorescence when all reaction centers are closed). The loss of PSII function was induced by changes on the acceptor side of PSII. Fast fluorescence decay showed a loss of PSII centers with bound Q_B, the secondary quinone acceptor of PSII, and a fast reoxidation kinetic of Q_A^-, the primary quinone acceptor of PSII, in the photoinactivated plants. No appreciable change could be observed in the amount of PSII centers with unbound Q_B and in Q_B-nonreducing PSII centers. Immunological studies showed that the contents of the D1 and D2 proteins of the PSII reaction center and of the 33-kDa protein of the water-splitting complex were diminished in the photoinhibited plants, and the occurrance of a new polypetide of 14 kDa that reacted with an antibody against the C-termius of the D1 protein. As shown by pulse-labelling experiments with [^{14}C]leucine both degradation and synthesis of the D1 protein were enhanced in the mineral-deficient plants when compared to non-deficient plants. A stimulation of D1-protein turnover was also observed in pH 3-grown plants, which were not inhibited at growth-light conditions. Obviously, stimulation of D1-protein turnover prevented photoinhibition in these plants. However, in the Mg- and Mg/S-deficient plants even a further stimulation of D1-protein turnover could not counteract the increased rate of photoinactivation.

Key words: D1-protein turnover – Mineral stress – Photoinhibition – Photosystem II – *Spinacia*

Introduction

Light energy drives photosynthesis but excess light is potentially damaging to the photosynthetic apparatus. The latter process is called photoinhibition and is manifested as an inhibition of photosynthetic electron transport at the level of PSII (Powles 1984; for recent reviews; Andersson and Styring 1991; Prasil et al. 1992). Interest in photoinhibition has increased as it has become clear that stress conditions can induce photoinhibition even at moderate light intensities (Kyle 1987; Ludlow 1987; Öquist et al. 1987). One of the important stress factors limiting photosynthesis is an imbalance in the supply of inorganic nutrients. Plants suffering a limited supply of magnesium, sulphur, phosphate, potassium or calcium are characterized by chlorosis of their leaves, which is enhanced under high light intensities (Marschner and Camak 1989; Dietz and Heilos 1990). Such plants might therefore be valuable for studying the molecular mechanism of stress-induced photoinhibition.

On a molecular level, photoinhibition is due to an impairment of PSII function (Andersson and Styring 1991; Prasil et al. 1992). Experimental results obtained

Dedicated to Professor Dr. Dres. hc. Achim Trebst on the occasion of his 65th birthday

Abbreviations: $amp_{(f,m,s)}$ = amplitude of the fast, (medium and slow) exponential component of fluorescence decay; F_m = yield of maximum fluorescenc when all reaction centers are closed; F_o = yield of intrinsic fluorescence at open PSII reaction centers in the dark; F_v = yield of variable fluorescence, (difference between F_m and F_o); LHC = light-harvesting complex; PFD = photon flux density; Q_A = primary quinone acceptor of PSII; Q_B = secondary quinone acceptor of PSII

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mostly with isolated thylakoids or PSII reaction centers suggest that light-induced damage to PSII may be caused by changes either on its acceptor site or on its donor site. According to the model of Styring (Styring et al. 1990; Andersson and Styring 1991), acceptor-site photoinactivation is induced when at high light intensities the capacity for CO_2 fixation becomes limiting and the plastoquinone pool is fully reduced. Under such conditions the operational frequency of the Q_B-binding site will be lowered and will lead to an overexcitation of the PSII reaction center and an increased double reduction of the primary quinone acceptor, Q_A (Setlik et al. 1990; Vass et al. 1992). After protonation, Q_A may then leave its binding site on the D2 protein. In these Q_A-depleted reaction centers charge separation will still occur (Virgin et al. 1988; van Mieghem et al. 1992), but cannot be stabilized. Instead, it induces the formation of the P_{680} chlorophyll triplet (Vass et al. 1992) which is thought to induce the degradation of the rapidly turning-over (Mattoo et al. 1984) D1 reaction-center polypeptide (Greenberg et al. 1987; Andersson and Styring 1991; Vass et al. 1992). Results from in-vivo systems such as intact algae seemed to indicate that a destabilization of the secondary quinone acceptor, Q_B, in its binding niche on the D1 protein is one of the first processes leading to photoinhibitory loss of PSII activity (Ohad et al. 1990; Prasil et al. 1992).

Donor-site photoinactivation is induced when electron transfer from the water-splitting system to P_{680} is slowed down (Andersson and Styring 1991; Prasil et al. 1992). It has been observed not only in chemically treated PSII membranes, where water-splitting was inactivated (Blubaugh and Cheniae 1990; Eckert et al. 1991; Jegerschöld and Styring 1991), but also in intact *Synechocystis* sp. PCC 6803 where the *psb* O gene encoding for the 33-kDa protein of the water-splitting system was deleted (Mayes et al. 1991). Under these conditions the lifetime of P_{680}^+ is increased (Blubaugh and Cheniae 1990; Shipton and Barber 1992). This species is also able to induce the breakdown of the D1 polypeptide, even under anaerobic conditions (Jegerschöld and Styring 1991; Shipton and Barber 1992).

Irreversibly photoinactivated PSII reaction centers can only be reactivated by a repair process, which depends on the synthesis of the D1 protein (Melis 1991; Andersson and Styring 1991; Kyle et al. 1984; Kyle 1987; Prasil et al. 1992). Therefore, the severity of photoinhibition in vivo not only depends on the photon flux density (PFD) and the probability of PSII overexitation, but also on the capacity of a plant to substitute for the degraded D1 protein by resynthesis (Kyle 1987).

Stress factors can enhance the degree of photoinhibition by various mechanisms. They might cause disturbances in the allocation of assimilates or induce the closing of stomata (Willenbrink et al. 1990). Both factors will lower the light-saturation point of photosynthesis and the rate of photoinactivation will increase. If the capacity to synthesise the D1 protein is still high enough, the increased rate of photoinactivation might be compensated for by an increased synthesis of D1 protein, and photoinhibition is avoided. However, stress conditions might also reduce the capacity for protein synthesis, so that photoinactivated PSII centers cannot be repaired sufficiently and photoinhibition is manifested (Kyle 1987). In this context we studied the combined effects of stress and light on the activity of PSII and on the light-dependent turnover of the D1 protein in mineral-deficient spinach plants.

Materials and methods

Plant material. Seeds of spinach (*Spinacia oleracea* L. cv. Matador) were germinated in soil. After two to three weeks the plants were transferred to 50-ml Erlenmeyer flasks containing a 1:10 (v/v) diluted nutrient solution, to clean the roots. After one week the plants were transferred to a 1:2 diluted nutrient solution, and after another two weeks they were transferred to full medium or to the deficient media. A sample of 50 plants, each with about six to eight leaves, was supported on a frame so that the roots were immersed in 25 l of nutrient solution. The solution containing $4 \cdot 10^{-3}$ M $Ca(NO_3)_2$, $6 \cdot 10^{-3}$ M KNO_3, 1 mM KH_2PO_4, $2 \cdot 10^{-3}$ M $MgSO_4$, $1 \cdot 10^{-3}$ M $MgCl_2$, $4.6 \cdot 10^{-5}$ M H_3BO_3, $1.1 \cdot 10^{-5}$ M $MnCl_2$, $7.65 \cdot 10^{-7}$ M $ZnSO_4$, $5.0 \cdot 10^{-7}$ M $CuSO_4$, $1.2 \cdot 10^{-5}$ M Na_2MoO_4, $3.2 \cdot 10^{-5}$ M $FeCl_2$ as EDTA complex. The nutrient solution was continuously aerated.

Imbalances in the nutrient supply were induced by acidifying the nutrient solution to pH 3.0 with H_2SO_4. The pH was adjusted once a week, by which time its pH had increased by about 0.5 units. Magnesium deficiency was induced by replacing $MgSO_4$ by K_2SO_4 and omitting $MgCl_2$ from the nutrient solution. A combined Mg and S deficiency was induced by omitting $MgSO_4$ and $MgCl_2$. The growth temperature was 12° C during the 15-h dark phase and 18° C during the 9-h light phase. The plants were grown at a PFD of 400–500 μmol photons $\cdot m^{-2} \cdot s^{-1}$.

Chlorophyll and protein determination. To determine the average chlorophyll concentrations, leaves (5 g fresh weight) were harvested and freeze-dried. After grinding 2 g of the dry leaves with $NaHCO_3$ in liquid nitrogen, chlorophyll was extracted with 2×5 ml 80% acetone. After filtration, chlorophyll concentrations were measured spectrometrically according to Arnon (1949). Protein was determined according to Bradford (1976).

Determination of starch content. For determination of the starch content, freeze-dried leaves were ground in liquid nitrogen. To 50 mg of the dry sample was added 80% boiling ethanol and the mixture was incubated for 2 min at 80° C. After cooling the sample on ice it was centrifuged for 2 min at 2000 rpm in an Eppendorf (Hamburg, Germany) 5415 C centrifuge. The sediment was again extracted with 80% ethanol, then with 40% ethanol and finally with boiling water. The sugar-free sediment was taken up in 1.25 ml 4 N HCl with 50% dimethyl sulfoxide and incubated for 1 h at 60° C. After cooling on ice and centrifugation at 14000 rpm for 4 min the supernatant was diluted up to 5 ml and adjusted to pH 4–5. After acidic hydrolysation the starch was degraded enzymatically with amyloglucosidase. The content of glucose was determined as reduction of NADP in a coupled enzymatic test with hexokinase and glucose-6-phosphate dehydrogenase (Boehringer, Mannheim, Germany).

Preparation of thylakoids. Spinach thylakoids were prepared according to Berthold et al. (1981) except that no bovine serum albumin was added.

Pulse-modulated chlorophyll fluorescence induction. The yield of intrinsic fluorescence at open PSII reaction centres in the dark (F_o) and the yield of maximum fluorescence when all reaction centres are closed (F_m) were determined by pulse-modulated chlorophyll fluorescence induction according to Schreiber et al. (1986) in leaf

discs using a PAM fluorometer (Walz, Effeltrich, Germany). The configuration consisted of the basic PAM 101 module, the emitter and detector unit ED-101, the module 102 with a PAM 102 L LED light source and the PAM 103 module with the high-intensity light source FL-103. The fluorometer and its measuring principles have been described previously (Schreiber et al. 1986). The PFD of the actinic light was 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the 500-ms saturating flashes and a PFD of 4000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The leaf discs were dark-adapted for 5 min to allow sufficient relaxation of the main quenching coefficients q_E (energy-dependent quenching) and q_p (photochemical quenching) (Rees et al. 1990). Increase of F_v/F_m after further dark adaptation was less than 2%. The intrinsic fluorescence F_o, when all reaction centers are open, was determined by applying only the measuring light (peak wavelength 710 nm); F_m was estimated from the fluorescence yield achieved on the addition of a 500-ms pulse of 4000 μmol photons $e \cdot m^{-2} \cdot s^{-1}$.

Decay of variable fluorescence. The dark decay of variable fluorescence after a single turnover flash was measured with the PAM fluorometer consisting of the basic PAM 101 module and the emitter/detector unit ED 101, the Xenon Single Turnover Flash XST 103 and its control unit PAM 103. The leaf disks were again dark-adapted for 5 min to allow maximal reoxidation of Q_A^- even in PSII centers not connected with the plastoquinone (PQ) pool (Chylla and Whitmarsh 1989). The value of F_o was determined by applying the measuring light. To reduce all of Q_A, plants were illuminated with a short flash (half peak time 8 µs) a high PFD of 20000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Sample were obtained at 25-µs intervals and stored using the DA-100 program (Kolbowski and Schreiber (1991); Walz, Effeltrich, Germany). The sampling rate was 40 kHz. The exponential analysis was performed using a program written by V. Ebbert (Biochemie der Pflanzen, Ruhr-Universität, Bochum, Germany). To deconvulate the fluorescence decay into its three exponential phases, first the slow phase was substracted from sample data by determining the amplitude after the end of the middle phase (25 ms). The amplitude and the lifetime of the middle phase were determined by fitting an exponential curve through the data points from the end of the first phase (3 ms). Its function was substracted from the first corrected data. As a result the purified fast phase was also fitted by an exponential analysis to obtain amplitude and lifetime. All amplitudes were related to F_o.

Immunological determination of thylakoid proteins. The relative contents of certain thylakoid proteins per mg chlorophyll were determined immunologically by Western-blotting. Thylakoids were first solubilized in 5% SDS, 15% glycerin, 50 mM Tris (pH 6.8) and 2% mercaptoethanol at room temperature for 30 min. After sonication with the microtip of a Branson sonicator (Schwäbisch-Gmünd, Germany), starch was centrifuge off. The polypeptides were separated by polyacrylamide gel (15%) electrophoresis according to Schägger et al. (1985). Proteins were then transferred onto nitrocellulose by electroblotting using a Bio-Rad (München, Germany) Transblot chamber for 3 h at 0.4 A in a cold room at −28° C. After saturation with 3% gelatine in Tris buffer (pH 7.5), the first antibody was allowed to react overnight at room temperature in 1% gelatine. After washing with Tris and 0.05% Tween-20, the second antibody (horseradish peroxidase; Bio-Rad) was allowed to react in 1% gelatine for 2 h. The membrane was treated with HRP colour developer (Bio-Rad) and 0.03% H_2O_2 to visualise the proteins. For detection of the D1 protein we used an antibody (Johanningmeier 1987) against part of the D1 protein (167–353) and β-galactosidase. The D2 protein was detected with an antibody against an oligopeptide containing amino acids 235–241 of the D2 protein. The antibody against the light-harvesting complex (LHC) proteins was a generous gift from U. Johanningmeier (Biochemie der Pflanzen, Ruhr-Universität, Bochum, Germany), and the antibody against the 33-kDa protein of the water-splitting system was obtained from N. Murata (Department of Regulation Biology, National Institute for Basic Biology, Okazaki, Japan).

Pulse labelling of the D1 protein. Freshly harvested spinach leaves (2 g) were cut into 1-cm^2 pieces. An aliquot of 10 ml of a 0.5 mM solution of [^{14}C]leucine with a specific radioactivity of 9.25 MBq was applied by vacuum infiltration (3 × 20 s). Synthesis of D1 protein was determined after 1 h incubation in the light (1000 µmol photons $\cdot m^{-2} \cdot s^{-1}$) according to Godde et al. (1991). To measure degradation of D1 protein it was labelled with [^{14}C]leucine at a PFD of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After 1 h the radioactivity was washed out by a 10 mM solution of cold leucine. Chloramphenicol was added to a final concentration of 800 $\mu g \cdot ml^{-1}$. The samples were illuminated for another 4 h at 6000 µmol photons $\cdot m^{-2} \cdot s^{-1}$.

Laser-densititometrical evaluation of Western blots and autoradiograms. The densitometry of Western blots and autoradiograms were performed with an Ultroscan XL Laser Densitometer (LKB, Uppsala, Sweden).

Results

Physiological parameters of the nutrient-deficient plants. To study PSII function and D1-protein turnover under stress conditions, we induced disturbancies in the nutrient supply to spinach plants by changing the composition of the growth medium. Spinach plants were either supplied with an acidic nutrient solution or with nutrient solutions where Mg alone or Mg together with S had been omitted. After eight to ten weeks exposure to the different media all deficient plants showed a drastic loss of chlorophyll when compared to the control (Table 1). The leaves of plants grown at low pH and of the Mg-deficient plants had only 50% of the chlorophyll content of the control plants. The Mg/S-deficient plants showed the highest loss of chlorophyll (85%). The starch content in all dark-adapted, deficient leaves was drastically increased (Table 1), the highest accumulation being shown by the Mg/S-deficient plants. This indicates that allocation of assimilates was probably disturbed in the plants under our mineral-stress conditions. All plants were disturbed in their water balance as indicated by the reduced ratio of fresh to dry weight.

Table 1. Physiological parameters of control plants and mineral-deficient spinach plants grown at pH 3 or on Mg-deficient or Mg/S-deficient media. Protein, chlorophyll (Chl) and starch contents were determined as described in *Materials and methods*. The values represent the arithmetic mean of at least three different determinants ± SD

	Control	pH 3	Mg deficient	Mg/S deficient
Protein/Chl $g \cdot g^{-1}$	6.5 ± 0.28	10.1 ± 0.71	12.7 ± 1.0	13.0 ± 0.5
Chl/DW $mg \cdot g^{-1}$	6.6 ± 0.30	3.1 ± 0.15	3.2 ± 0.2	0.9 ± 0.02
Starch/DW $mg \cdot g^{-1}$	0.6 ± 0.02	10.4 ± 3.00	10.1 ± 1.1	16.3 ± 2.3
FW/DW	8.1 ± 0.60	5.8 ± 0.40	7.3 ± 0.7	6.3 ± 0.7

Activity of PSII as measured by chlorophyll fluorescence. In order to test the effects of mineral deficiency on the activity of PSII and on photoinhibition in vivo, we used leaf disks to study the chlorophyll-fluorescence parameter F_v/F_m, which reflects the quantum yield of PSII photochemistry (Krause and Weis 1991). The spinach plants tested were exposed to the different growth media for eight to ten weeks. As shown in Fig. 1, leaves of non-deficient control plants which had been dark-adapted for 15 h had a high F_v/F_m ratio, indicating that all PSII centers were functionally active. The ratio remained stable after 3 h illumination of the plants with growth light of 400 µmol photons·m^{-2}·s^{-1}. Dark-adpated pH 3-grown plants had a F_v/F_m ratio similar to the control, again indicating functional activity of PSII. The ratio stayed relatively stable after 3 h illumination.

In dark-adapted Mg-deficient plants the F_v/F_m ratio was somewhat lower than in the control, but still high enough to assume functional acitivity of PSII. It declined slightly, but significantly during the illumination at 400 µmol photons·m^{-2}·s^{-1}, indicating a photoinduced loss of PSII function. In plants grown on an Mg/S-deficient medium the 15-h dark-adapted leaves showed a decreased F_v/F_m ratio when compared to the control. During illumination, loss of PSII activity was accelerated and F_v/F_m declined further. Interestingly, plants deficient only in Mg could recover almost completely in the 15-h dark phase. Such a complete recovery up to F_v/F_m values around 0.8 could not be detected in the Mg/S-deficient plants.

The light-induced loss of variable fluorescence seen in the Mg/S-deficient plants was mostly due to an increase in F_o (data not shown). In dark-adapted Mg/S-deficient plants, F_o was 27.1±0.57, whereas the value found for the non-deficient control plants was 17.0±2.2. After illumination, F_o in the Mg/S-deficient plants increased up to 37.2±13.1. In the control plants it remained stable at 17.0±2.2. There was no significant difference in F_m between control and nutrient-deficient Mg/S either in the light or in the dark. In the Mg-deficient plants the loss of F_v/F_m was due to a loss of F_m. In the dark-adapted Mg-deficient plants, F_m was 86.6±12.4, after 3 h of illumination it decreased to 71.3±10.4. The value of F_o was 18.7±2.0 and increased slightly to 21.4±1.1 after the light period.

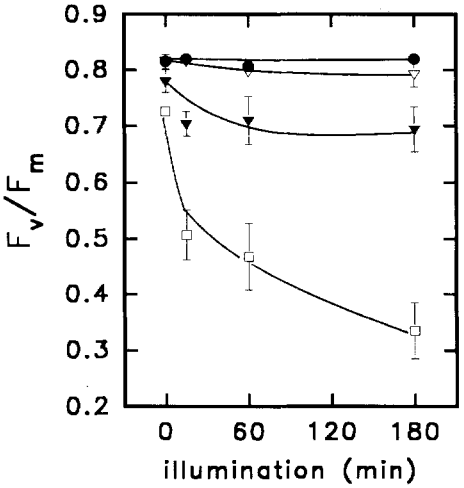

Fig. 1. Changes in F_v/F_m, as determined by pulse-modulated chlorophyll fluorescence, in leaf disks of control (●) and mineral-deficient spinach growth either at pH 3 (▽), or supplied with an Mg (▼)- or an Mg/S (□)-deficient medium. Data were obtained during the first 3 h of illumination at a PFD of 400 µmol·photons·m^{-2}·s^{-1} after a 15-h period of complete darkness. F_o was determined by applying measuring light; F_m was determined from the fluorescence yield achieved upon the addition of a 500-ms pulse of 4000 µmol·photons·m^{-2}·s^{-1}

Flash-induced fast fluorescence decay. Photoinactivation in the nutrient-deficient plants was concomitant with changes in the reoxidation kinetics of Q_A^-, as followed by fluorescence-relaxation measurements after a saturating single turnover flash in the intact leaf system. In this method, approximately all of Q_A is reduced due to the high PFD of the single turnover flash. The decline of fluorescence reflects the reoxidation of Q_A^-. Normally, in a non-photoinactivated leaf, fluorescence decays exponentially in three phases fast, medium and slow, which are characterized by the amplitudes (amp_f, amp_m

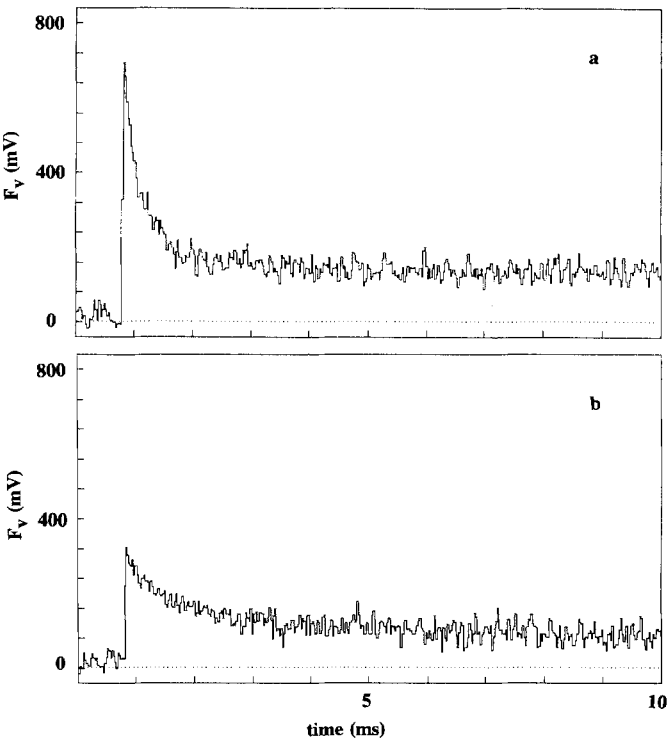

Fig. 2a, b. Typical kinetics of Q_A^- reoxidation followed by fast fluorescence decay after a single turnover flash (20000 µmol photons·m^{-2}·s^{-1}, half-peak time = 8 µs) in control (**a**) and Mg/S-deficient (**b**) spinach plants. The leaves had been illuminated for 3 h. The curves were plotted with the same scale. $F_v = 0$ is identical to F_o. F_o of the control was 366 mV, and F_o of the deficient plants was 1196 mV. F_v/F_m was 0.666 of the control and 0.221 for the deficient plant, respectively. Amp_f related to F_o for the control plants was 1.51, for the deficient plants 0.12; amp_m was 0.23 for the control and 0.11 for the deficient plants; amp_s was 0.26 for the control and 0.14 for the deficient plants

Table 2. Kinetics of fast fluorescence decay after a single turnover flash in dark-adapted or 3-h-illuminated leaves from spinach grown for eight to ten weeks on nutrient medium at pH 3, or on Mg- or Mg/S-deficient media. The data are based on three different determinations ± SD

Plant material	F_v/F_m	amp_f	amp_m	amp_s
Control plants				
Dark adapted	0.670 ± 0.002	1.54 ± 0.014	0.12 ± 0.06	0.37 ± 0.001
3 h illuminated	0.694 ± 0.002	1.76 ± 0.080	0.24 ± 0.03	0.24 ± 0.010
pH 3				
Dark adapted	0.657 ± 0.040	1.43 ± 0.330	0.18 ± 0.02	0.36 ± 0.020
3 h illuminated	0.666 ± 0.020	1.55 ± 0.060	0.09 ± 0.06	0.37 ± 0.030
Mg deficient				
Dark adapted	0.693 ± 0.004	1.65 ± 0.030	0.22 ± 0.09	0.39 ± 0.020
3 h illuminated	0.652 ± 0.002	1.43 ± 0.010	0.18 ± 0.06	0.26 ± 0.090
Mg/S deficient				
Dark adapted	0.342 ± 0.008	0.31 ± 0.005	0.10 ± 0.03	0.12 ± 0.004
3 h illuminated	0.302 ± 0.003	0.17 ± 0.020	0.12 ± 0.02	0.15 ± 0.030

and amp_s, respectively) of these three exponential components (Robinson and Crofts 1983; Cao and Govindjee 1990; Krause and Weis 1991; Fig. 2a). The amplitude amp_f reflects PSII centers with bound Q_B which oxidizes Q_A^- very fast with a half-life time around 200 µs. In dark-adapted non-deficient control leaves, 76% of all PSII centers were characterized by fast Q_A^- reoxidation (Table 2). The medium phase amp_m reflects PSII with an empty Q_B side where oxidation of reduced plastoquinone by the cyt b_6/f complex and its rebinding to the Q_B-binding niche on the D1 protein is rate limiting (half-life time 1–2 ms). In the dark-adapted control leaves only 6% of PSII centers had an empty Q_B side. The third and slowest phase, amp_s, with a half-life time of 2 s is due to PSII not connected with the plastoquinone pool, so called Q_B-nonreducing centers, where reoxidation of Q_A^- takes place via the S_2-state of the water-splitting system (Chylla and Whitmarsh 1989). In dark-adapted control plants, 18% of all PSII belonged to these Q_B-nonreducing centers.

By plotting all measured F_v/F_m ratios against the amplitudes of the three exponential phases of fluorescence decay, it could be shown, that F_v/F_m was to a great part determined by amp_f, which reflects the number of PSII centers with bound Q_B (Fig. 3). No correlation could be found between F_v/F_m and amp_m or amp_s in the mineral-deficient leaves. It should be mentioned that F_m measured after a single turnover flash is always lower than the value found by the method of Kautsky fluorescence induction, since fluorescence is still quenched by oxidized plastoquinone (Krause and Weis 1991).

The dark-adapted control plants had a high number of PSII centers with fast reoxidation of Q_A^- (Fig. 2a, Table 2) as represented by an amp_f of 1.54 ± 0.014. After 3 h illumination, amp_f even increased (Table 2), raising F_v/F_m from 0.670 ± 0.002 to 0.694 ± 0.002. The number of PSII centers with unbound Q_B, reflected by amp_m, increased slightly during illumination. On the other hand we observed a slight loss of slow Q_B-nonreducing PSII centers. These centers seemed to be transformed into active Q_B-reducing centers, either belonging to amp_f or

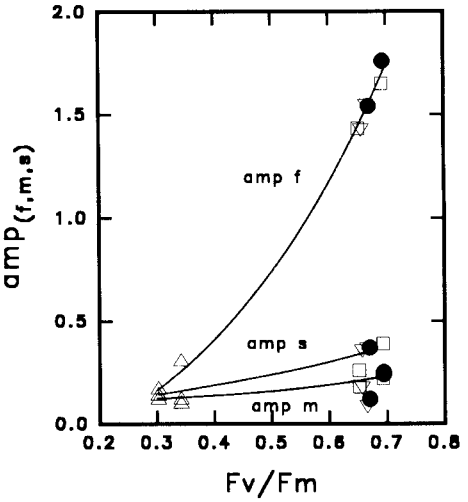

Fig. 3. Correlation between F_v/F_m and the amplitudes amp_f, amp_m and amp_s of the three exponential phases of fast fluorescence decay after a single turnover flash. The amplitudes were standardized to F_o. The data presented were taken from Table 2, and are for control (●), pH 3-grown (▽), Mg-deficient (□) and Mg/S-deficient (△) spinach plants. F_o was determined by applying the measuring light; F_m was determined from the fluorescence yield achieved by the single turnover flash

amp_m, as has already been found by Guenther et al. (1990).

In the pH 3-grown plants, amp_f was slightly lower than in the control, with a value of only 1.43 ± 0.33 in the dark-adpated leaves and 1.55 ± 0.06 in the illuminated leaves (Table 2). Again, the light-induced increase in amp_f resulted in a rise of F_v/F_m. The Mg-deficient plants had a very high number of fast PSII centers, with an amp_f of 1.65 ± 0.03 even in the dark-adapted leaves, together with a high F_v/F_m ratio (Table 2). However, during illumination amp_f declined, resulting in a lower F_v/F_m. Obviously, a significant number of PSII centers with fast Q_A^- reoxidation was lost during illumination. This confirms the results seen in the Kautsky fluorescence induction. The dark-adpated Mg/S-deficient plants already had a very low amp_f ratio of 0.31 ± 0.005,

which decreased further to 0.17 ± 0.02 in the light (Fig. 2b, Table 2), thus confirming in principle confirm the results shown in Fig. 1. The differences in the F_v/F_m ratios of dark-adapted leaves shown in Table 2 and Fig. 1 might be due to the different methods of determination. On the other hand, they might also be caused by differences in the ages of the plant material. No light-induced change could be observed in amp_m, which stayed stable around 0.12. Also, amp_s stayed relatively stable.

This, light-induced loss of F_v/F_m in the mineral-deficient plants could be ascribed to a decrease in PSII centers with bound, oxidized Q_B and fast reoxidation kinetics of Q_A. This has already been found under classical photoinhibitory conditions, where intact leaves or thylakoid preparations has been exposed to excess light flux densities (Briantais et al. 1992; Godde et al. 1992; Vass et al. 1992). In non-deficient control plants the number of PSII centers with a fast fluorescence decline was found to rise upon illumination, probably due to a light activation of Q_B-nonreducing centers with a slow reoxidation of Q_A^-.

Immunological determination of PSII and LHCII polypeptides. Photoinactivation of PSII is known to induce the breakdown of the D1 protein (Andersson and Styring 1991; Prasil et al. 1992). In systems without protein biosynthesis this can be seen directly as a loss in D1-protein content. In intact plant systems the correlation between D1-protein content and activity of PSII is more complex (Smith et al. 1990; Lütz et al. 1992).

To study the effects of chronic stress conditions on the number of PSII reaction centers the contents of D1 and D2 protein per chlorophyll in the different leaves were determined by Western-blotting. After eight weeks exposure, leaves of pH 3-grown plants still had the same contents of D1 and D2 protein (Fig. 4) per unit of chlorophyll as the control plants, as determined by Western-blotting, showing that the number of reaction-center polypeptides was not diminished. A slight loss of D1-protein content of 20% and 30% could only be shown in leaves of Mg- and Mg/S-deficient plants by laser densitometry of the Western blots. The D2 protein was diminished to about the same extent. No further reduction of PSII centers or D1 protein could be observed when the plants were illuminated for 3 h. Interestingly, a 14-kDa polypeptide occurred in the Mg- and Mg/S-deficient plants, and this could be identified with our antibody as a C-terminal degradation product of the D1 protein. No fragment of the D2 protein could be detected. Prolonged exposure of plants to the deficient media aggravated the decrease in the contents of D1 and D2 proteins on a chlorophyll basis. After 12 weeks, the pH 3-grown plants contained only 60% D1 and 80% D2 protein (data not shown). In Mg-deficient plants the content of these polypeptides decreased down to 20% of the control and in the Mg/S-deficient plants it was around 20–30%. Again no light-induced loss of D1 or D2 protein could be detected. The 14-kDa fragment could now be detected even in the illuminated, pH 3-grown plants.

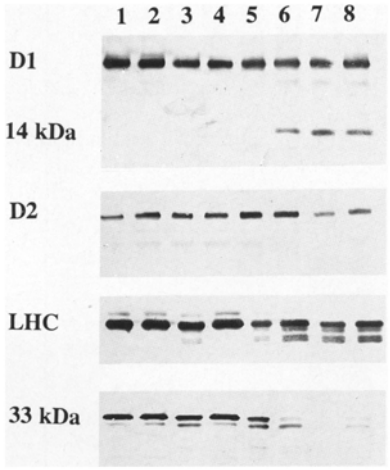

Fig. 4. Contents of D1 and D2 proteins, LHCII protein and 33-kDa protein as determined by Western-blotting in control and mineral-deficient spinach plants. Each row represents a different zone cut out of immunoblots. Rows labelled *D1* and *14 kDa* were developed with anti-D1 antibodies, the row labelled *D2* with anti-D2 antibodies, the row labelled *LHC* with anti-LHCII protein and the row labelled *33 kDa* with anti-33 kDa antibodies. *Lane 1* 15-h dark-adapted control plants; *lane 2* 3-h-illuminated control plants; *lane 3*, dark-adapted pH 3-grown plants; *lane 4*, illuiminated pH 3-grown plants; *lane 5*, Mg-deficient plants, dark adapted; *lane 6*, Mg-deficient plants, illuminated; *lane 7*, Mg/S-deficient plants, dark adapted; *lane 8*, Mg/S-deficient plants, illuminated. Each lane was loaded with equal amounts of chlorophyll (2.5 μg)

The protein complexes of LHCII bind more than 40% of the chlorophyll (Melis et al. 1991) and the ratio of protein complex to chlorophyll should stay constant, even in yellowing plants. Indeed, no appreciable change in the immunologically determined content of LHCII polypeptides per unit chlorophyll could be detected in the mineral-deficient plants (Fig. 4, row LHC). Interestingly, in Mg- and Mg/S-deficient plants we observed smaller polypeptides reacting with our antibody against LHC proteins. We assume that these bands were degradation products of the mature proteine, indicating that the stability of LHCII polypeptides was also decreased. The reduction in PSII reaction-center polypeptides on a chlorophyll basis therefore reflects a decreased ratio of PSII reaction centers per LHCII.

Beside the changes in the chlorophyll-containing protein complexes, we also observed a very early and substantial decrease in the 33-kDa protein of the water-splitting system (Fig. 4). Interestingly, this protein was almost completely absent in thylakoids isolated from eight-week-old Mg/S-deficient plants and drastically diminished in plants suffering only from Mg deficiency. Plants grown for eight weeks at pH 3 had the same amount of 33-kDa protein as the control. In all plants we could detect a smaller polypeptide reacting with the antibody against the 33-kDa polypeptide. This band increased relative to the band of the mature protein in the deficient plants. It should be mentioned that the 33-kDa protein was already diminished by 70% in light-adapted plants exposed for six weeks on Mg/S-deficient medium. After 12 weeks exposure the protein was no longer detectable in any of the deficient plants.

Turnover of the D1 protein. To test whether the turnover of the D1 protein was altered under the different stress conditions, synthesis of the D1 protein was measured in vivo by pulse-labelling intact leaves with [^{14}C]leucine at a PFD of 1000 µmol·m^{-2}·s^{-1}, to guarantee a sufficient labelling of the D1 protein, and compared with the actual D1-protein content determined by Western blotting. Figure 5 shows the incorporation of [^{14}C]leucine into the D1 protein in comparison with its content immunologically determined after the light period. It can be seen that the incorporation of the radioactive amino acid was relatively low in the control plants (Fig. 5b, c), which had the highest content of the D1 protein (Fig. 5a). It was substantially stimulated by about a factor of 2–3 in the Mg- and Mg/S-deficient plants. In these plants the D1 content was appreciably diminished when compared to the control. This means that the degradation of the D1 protein in the deficient plants was also accelerated. Interestingly, synthesis of D1 protein was also stimulated in the pH 3-grown plants, which did not show any photoinhibitory loss of PSII activity at growth light conditions. However, the content of the D1 protein in these plants was only slightly diminished when compared to the control. Thus, we assumed that in these plants the degradation of the D1 protein was higher than in the control, but lower than in the other deficient plants.

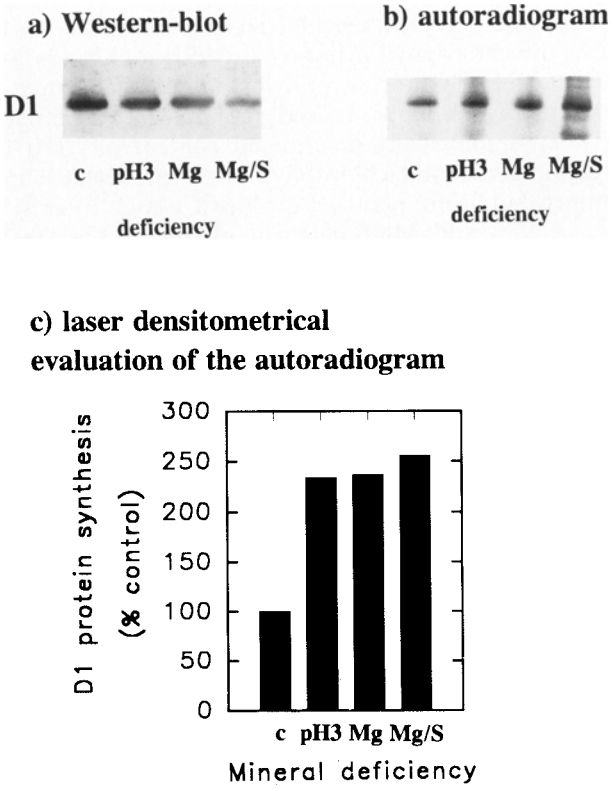

Fig. 5a–c. Incorporation of [^{14}C]leucine into the D1 protein of control (*lane 1*), pH 3-grown (*lane 2*), Mg-deficient (*lane 3*) and Mg/S-deficient spinach (*lane 4*) plants after 1 h illumination at 1000 µmol photons·m^{-2}·s^{-1}. The SDS-gels were loaded with equal amounts of chlorophyll (2.5 µg). **a** Western-blot, **b** autoradiogram, **c** laster-densitometrical evaluation of the autoradiogram

This could be confirmed by following the degradation of D1 protein in a subsequent chase period with cold leucine in the presence of chloramphenicol. To obtain a sufficient turnover of the D1 protein in the intact leaf system it was necessary to work at higher irradiances of 6000 µmol·photons·m^{-2}·s^{-1}. Preceding experiments (data not shown) had shown that a significant decrease in the amount of radioactivity could only be observed at high PFDs. As is shown in Fig. 6, after 1 h the pool of D1 protein in all plants was equally labelled. This was due to the fact that at high PFDs degradation of the D1 protein in the deficient plants was much more stimulated than at the lower PFDs under which D1-protein synthesis had been measured. After another 4 h of illumination the radioactivity incorporated into the control leaves had not appreciably decreased which reflects the relatively high stability of the D1 protein in the non-photoinhibited control. Another reason for this result might be the use of chloramphenicol, which has been shown to act as an acceptor of PSI and might therefore influence the level of PSII photoinactivation (Okada et al. 1991). However, in the Mg- and Mg/S-deficient plants the radioactivity in the D1 protein decreased to about 20%, showing that degradation of D1 protein was substantially stimulated in the photoinhibited leaves. Also the pH 3-grown plants showed almost a twofold increase in D1-protein degradation, confirming the result deduced from the data for D1-protein synthesis. Both experiments, synthesis of the D1 protein,

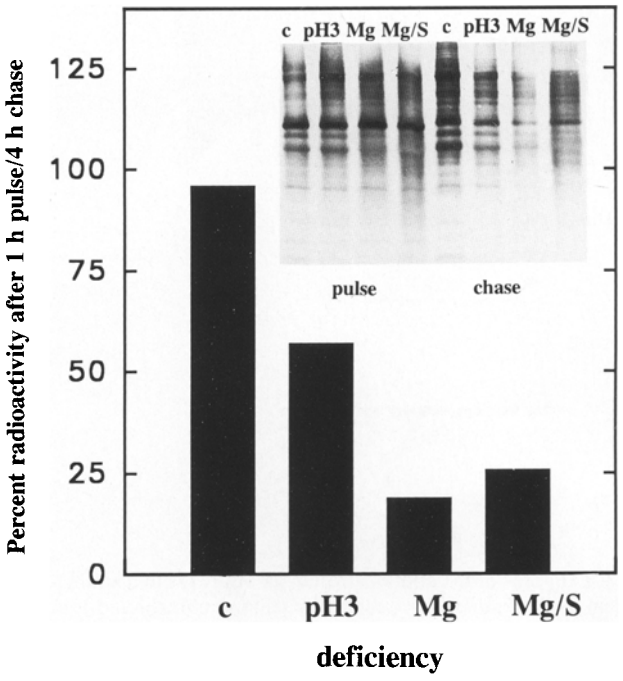

Fig. 6. Degradation of the D1 protein in control and mineral-deficient spinach plants. The D1 protein was labelled with [^{14}C]leucine for 1 h at 6000 µmol photons·m^{-2}·s^{-1}. To inhibit protein synthesis chloramphenicol was added and the leaves were further illuminated for 4 h. *Histogram:* laser-densitometrical evaluation. *Inset:* autoradiograms of gels after labelling of D1 protein for 1 h (four *left-hand* gels) and after inhibition of protein synthesis and illumination for 4 h (four *right-hand* gels)

measured at 1000 μmol·photons·m^{-2}·s^{-1}, and the pulse-chase experiment, measured at 6000 μmol·photons·m^{-2}·s^{-1}, showed that D1-protein turnover was accelerated in the plants suffering from mineral stress. Thus, it seems most likely that turnover was also stimulated under the growing conditions. This assumption is supported by the occurrence of the 14-kDa degradation product already found in the Mg- and Mg/S-deficient plants at growth light conditions.

Discussion

Plants suffering from certain mineral-stress conditions such as N, P, Mg and S deficiency are characterized by a light-enhanced loss of chlorophyll and, on the other hand, by an increase in the content of starch and probably also of soluble sugars (Marschner and Camak 1989; Dietz and Heilos 1990). Chlorosis and starch accumulation were similarly observed in plants grown in an acidic medium, or in Mg-deficient or Mg/S-deficient media. It has been suggested that the reduced growth observed in mineral-deficient plants is due to disturbances in CO_2 fixation and assimilate partitioning, and not primarily limited by photosynthetic reactions (Marschner and Camak 1989; Dietz and Heilos 1990; Henley et al. 1991).

Our results show that photosynthetic electron transport is also affected under chronic mineral stress. Plants suffering from Mg deficiency alone or in combination with S deficiency showed a light-induced loss of PSII function as measured by F_v/F_m at growth light conditions of 400 μmol·photons·m^{-2}·s^{-1}. It seemed to be loosely correlated with the loss of chlorophyll and the increased starch content, since the strongest photoinhibition at growth light conditions was found in the Mg/S-deficient plants with the highest starch and the lowest chlorophyll contents. Thus, stress conditions did increase the probability of photoinhibition, as has been often proposed (Kyle 1987). However, substantial photoinhibition under chronic stress conditions was only observed when the chlorophyll content was already reduced by more than a half.

The loss of PSII activity found in Mg- and Mg/S-deficient plants was due to alterations of electron flow at the acceptor side of PSII, as could be shown by fluorescence-decay measurements. The loss of PSII activity found in the photoinhibited leaves could be ascribed to a decrease of PSII centers with bound oxidized Q_B and a fast reoxidation of Q_A^- (Robinson and Crofts 1983; Cao and Govindjee 1990; Krause and Weis 1991), as has already been found under classical photoinhibitory conditions (Briantais et al. 1992; Godde et al. 1992; Vass et al. 1992). Photosystem II centers with unbound Q_B or Q_B nonreducing centers were not lost in the deficient plants when compared to the control. This confirms the results of Krause et al. (1990) showing that Q_B-nonreducing centers cannot be photoinhibited.

However, the specific loss of fast Q_A^- oxidizing PSII centers seen in the photoinhibited Mg/S-deficient plants does not tell very much about the mechanism of PSII photoinactivation. It might be due to a reduced affinity of plastoquinone for the Q_B-binding niche (Etienne et al. 1990; Briantais et al. 1992). On the other hand, it is also possible that the decreased ratio of amp_f to amp_m might just reflect an increase in the reduction state of the plastoquinone pool. It has recently been shown that even in higher plants the plastoquinone pool can be reduced in the dark (Asada et al. 1992; Groom et al. 1993), possibly due to the activity of an NAD(P)H-plastoquinone oxidoreductase (Godde 1982). The higher level of the plastoquinone reduction state, even found in dark-adapted leaves, might finally be caused by the accumulation of starch, which is known to lead to an increase in the NADPH/NADP ratio (Stitt 1986; Dietz and Heilos 1990). These conditions should slow down reoxidation of plastoquinone in the light and possibly also induce its reduction in the dark and, in this way, should favour photoinactivation of PSII via the acceptor side.

However, the loss of the 33-kDa polypeptide binding to the thylakoid membrane of the deficient plants might have accelerated loss of PSII function. Although this protein is not essential for electron transfer from water to P_{680}, malfunction of the water-splitting system is known to enhance photoinhibition of PSII (Blubaugh and Cheniae 1990; Eckert et al. 1991; Jegerschöld and Styring 1991; Mayes et al. 1991). The reasons for the diminished binding of the 33-kDa protein are not completely understood. We assume that the high turnover of the D1 protein found in the deficient plants prevents a sufficient reassembly between the water-splitting system and the PSII reaction center. On the other hand, the pH in the thylakoid lumen might be so low that it causes the dissociation of the 33-kDa protein (Shen and Katoh 1991). Although it is known from in-vitro systems that the degradation products of the D1 protein depend on the mechanisms of photoinactivation (Barbato et al. 1991; De Las Rivas et al. 1992; Virgin et al. 1992), the observed 14-kDa degradation product could not be assigned to any one of the different mechanisms.

Photoinactivation as it was observed in the Mg- and Mg/S-deficient plants is known to accelerate the turnover of the D1 protein (Kyle et al. 1984; Andersson and Styring 1991; Prasil et al. 1992; Virgin et al. 1992). Both degradation and synthesis of the "highly turning over" D1 protein (Mattoo et al. 1984) are part of a repair mechanism for PSII necessary for a recovery from photoinactivation (Andersson and Styring 1991; Melis 1991; Prasil et al. 1992). The degree of actual photoinhibition should therefore not only be determined by overexitation and damage to the PSII reaction center but also by the rate of its repair. We found by radioactive pulse-labelling experiments with [^{14}C]leucine, that the D1 protein was synthesized faster in all deficient plants. Since the content of D1 protein in these plants was comparable to or even lower than in the control, we have to conclude that the degradation of the D1 protein was also stimulated. This could be confirmed by the pulse-chase experiments. Although both experiments had to be carried out for technical reasons under unphysiological conditions and at higher PFDs than that for growth, it seems likely that the D1 turnover was also accelerated under growth light conditions, which is substantiated by the

occurrence of the 14-kDa degradation product of D1 protein.

Interestingly, the stimulation of D1-protein turnover, both synthesis and degradation, was already observed in the pH 3-grown plants which were not photoinhibited at growth light conditions. If indeed degradation of D1 protein is controlled by PSII overexitation, as has been proposed (Andersson and Styring 1991; Prasil et al. 1992), the increased turnover of the D1 protein indicates that damage to the PSII reaction center had already occurred before it became detectable as inhibition of PSII activity. However, in these plants, D1-protein turnover seemed to be high enough to compensate for the increased damage to PSII and a measurable photoinactivation could be prevented. Thus, turnover of the D1 protein reacts much faster to stress conditions than PSII activity itself. A similar stimulation of D1-protein turnover has also been found in leaves of ozone-fumigated spruce trees and damaged trees from outdoors, although PSII functionality was not disturbed (Godde 1992; Godde and Buchhold 1992).

In the Mg-deficient plants, and especially in the Mg/S-deficient plants, turnover of the D1 protein was not high enough for a sufficient repair of PSII function, and photoinactivation of PSII was manifested. However, no loss of D1 protein was detectable after 3 h illumination at the growth PFD under the two different mineral conditions, again showing that light-induced loss of PSII function in higher plants is not identical to the loss of D1 protein (Virgin et al. 1988; Aro et al. 1992; Godde et al. 1992). It also confirms that degradation and synthesis of the D1 protein are in vivo strongly coupled, as has been shown by Adir et al. (1990). On the other hand, one has to assume that, in contrast to the hypothesis of Kyle (1987), not only D1-protein synthesis but also D1-protein turnover, both synthesis and degradation, is limiting PSII recovery. In the long run, however, synthesis of the D1 protein must have been outbalanced by its degradation, leading to the the loss of D1 protein and of the whole PSII reaction center, which is known to be degraded when D1 protein is missing (Schuster et al. 1988). Such a loss of PSII reaction-center polypeptides has already been observed under other chronic conditions (Lütz et al. 1992). Since the ratio of LHCII polypeptides to chlorophyll is fairly stable, the specific loss of PSII reaction-center polypeptides on a chlorophyll basis indicates that in nutrient-deficient plants there is an excess of LHCII proteins per PSII reaction-center. This might indicate that degradation of the reaction center precedes degradation of the light-harvesting system.

Our results provide evidence that stress factors stimulate photoinhibition by two mechanisms. They enhance the probability of PSII overexitation, most likely by raising the ratio of reduced versus oxidized quinone. The increased damage to PSII results in a stimulation of D1-protein turnover. In a certain range the increased turnover of the D1 protein is able to counteract damage to PSII and prevents functional loss of PSII activity, as could be seen in the pH 3-grown plants. However, when the D1-protein turnover becomes limiting and cannot be stimulated any further, as was the case in the Mg/S-deficient plants, damage to PSII is manifested.

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