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Photoinhibition and Recovery of Photosynthesis in *psbA* Gene-Inactivated Strains of Cyanobacterium *Anacystis nidulans*[1]

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ABSTRACT

The susceptibility of photosynthesis to photoinhibition and the rate of its recovery were studied in cyanobacterium *Anacystis nidulans* strain R2 and its two *psbA* gene-inactivated mutants R2S2C3 and R2K1. Changes in the fluorescence kinetics at 77K as well as the rate of O_2 evolution were measured when cells were exposed to high photosynthetic photon flux densities in the range of 0 to 2,000 micromoles per square meter per second. The R2S2C3 mutant has an active *psbAI* gene highly expressed under low and normal light intensities, whereas R2K1 possesses *psbAII* and *psbAIII* genes highly expressed under very high light intensities. The level of overall susceptibility of photosynthesis to photoinhibition was more pronounced in the wild type and the mutant R2S2C3 than in the mutant R2K1, especially at higher light intensities. In contrast, all three strains showed an increased but similar sensitivity to photoinhibition after addition of the translational inhibitor streptomycin; mutant R2K1 being slightly less sensitive at lower light intensities. The result is interpreted as demonstrating similar intrinsic susceptibility to photoinhibition of the two different forms of the D1 protein, form I and form II, encoded by the *psbAI* and *psbAII/psbAIII* genes, respectively. The increased resistance to photoinhibition of the R2K1 mutant was ascribed to an approximately 3 times higher rate of recovery than the wild type and the mutant R2S2C3. On the basis of our experiments we conclude that the susceptibilities to photoinhibition of the *Anacystis nidulans psbA* genes mutants studied are regulated mainly by modifying the rate of repair, *i.e.* the rate of turnover of the D1 protein.

Under high light conditions photoinhibition of photosynthesis may occur in higher plants as well as in algae (18, 19). The primary site of photoinhibition has been suggested to be located within the D1 protein (also called Q_B- or herbicide-binding protein) to which the PSII reaction center is bound (1, 4, 8, 9, 13, 24). However, the molecular mechanism of photoinhibition and the significance of the degradation of the D1 protein in relation to photoinhibition is still debated (2). Photosynthetic organisms are able to recover from photoinhibition of photosynthesis because of a repair process that occurs when organisms are placed again under favorable growth conditions (13, 14, 20, 21).

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The process of photoinhibition and recovery in the cyanobacterium *Anacystis nidulans* has been extensively investigated in our laboratory (10, 11, 20, 21). We have previously shown that *A. nidulans* responds to photoinhibition in a way similar to higher plants (20). The rate of photoinhibition and the rate of recovery of photosynthesis were dependent on the growth light intensity—the level of photoinhibition being inversely proportional and the level of recovery directly proportional (20, 21). It also has been found that the rate of the synthesis of the D1 protein plays a major role both in the susceptibility of photosynthesis to photoinhibition and during the recovery process (11, 21).

The D1 protein is coded for by the *psbA* gene. It is well known that the genome of *A. nidulans* contains three *psbA* genes—*psbAI*, *psbAII*, and *psbAIII* (3, 6). These can be inactivated on the *Anacystis* chromosome, singly or in pairs, and it was shown that each gene alone provides sufficient protein product to support normal photosynthesis (6). Wild type strain R2 has been described by Golden *et al.* (6) as having all three *psbA* genes active, although the *psbAI* normally provides about 94% of the *psbA* gene transcripts. These genes encode two forms of the D1 protein—form I and form II. Both forms of the D1 protein differ at 25 residues, 12 of which are the first 16 amino acids of the protein as predicted by three 1080 base-pair *psbA* genes (6). Mutant R2S2C3 has its *psbAII* and *psbAIII* genes inactivated by the insertion of gene cassettes encoding resistance to spectinomycin and chloramphenicol. Its only active *psbA* gene is *psbAI*, which in wild-type cells is highly expressed under low and normal light conditions and produces form I of the D1 protein (6, 22). Mutant R2K1 has the *psbAI* gene inactivated by insertion of a kanamycin-resistant cassette and possesses *psbAII* and *psbAIII* genes. These genes are highly expressed under very high light conditions and both produce form II of the D1 protein (6, 22).

We have attempted to determine whether the differential *psbA* gene expression reported in high and low light grown *A. nidulans* provides a means to modify the reaction center of PSII in concert with the light regime, thereby providing increased resistance to photoinhibition under a high light conditions. In the present work we have characterized the susceptibility of photosynthesis to photoinhibition in *A. nidulans* strain R2 and its *psbA* gene-inactivated mutants R2S2C3 and R2K1. Since repair of photodamage provides a means to avoid net photoinhibition (21) we have also investigated the

recovery kinetics after photoinhibition. We have used low temperature fluorescence kinetics and emission spectra measurements as well as measurements of oxygen evolution. The three strains have also been characterized with respect of growth and pigment synthesis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Anacystis nidulans strain R2 and two *psbA* gene-inactivated mutants (strains R2S2C3 and R2K1) described by Golden *et al.* (6) were grown in batch cultures in an inorganic medium as in (23) with one modification. Iron content in the medium was doubled in order to prevent disturbances in the Chl synthesis observed for these strains when grown in a standard medium. The temperature was kept at 38°C and the cultures were exposed to continuous PPFD of 50 μmol m^{-2} s^{-1} as measured with a Li-Cor quantum radiometer (Lambda Instruments, Lincoln, NE). Light sources were Sylvania Hi-Light PAR 38 lamps, 120W/ 240-250V; 5% CO_2 in air was flushed over the cultures to avoid changes in antenna size due to low inorganic carbon content in the medium. The cultures used for photoinhibitory treatments were always in the logarithmic phase of growth, diluted daily in order to minimize effects of self-shading.

Spectroscopic Measurements

Cell densities were determined from absorbance measurements at 750 nm (Shimadzu MPS 2000 spectrophotometer). Number of cells per mL culture was estimated as in Lönneborg *et al.* (10). For the estimation of phycocyanin and Chl *a* content, absorption spectra between 550 and 750 nm were recorded on Shimadzu MPS 2000 spectrophotometer. The amounts of phycocyanin, Chl *a* and A_{625}/A_{678} ratios were calculated as described before (12).

Growth Characteristic Experiments

During the growth experiments all three strains were grown continuously without dilution. Culture samples containing about $3 \cdot 10^8$ cells were diluted with growth medium to a final volume of 150 mL. Light, temperature and air conditions were the same as described above. Aliquots of cultures were withdrawn at different time points (as indicated in figures) in order to measure cell density and pigment content. The measurements were repeated until cultures showed the symptoms of total cell degradation (approximately after 120 h of continuous growth).

Photoinhibitory Treatment and Reactivation

The cells were harvested by centrifugation at 3000*g* for 5 min, washed once with growth medium, and resuspended to a final concentration of 2 μg Chl/mL. The inorganic carbon from 10 mM $NaHCO_3$ stock solution was added to a concentration of 100 μM. All operations were carried out at 38°C. The tubes containing algal suspensions were placed in a glass water bath at 38°C. The light source was a halogen lamp (Osram, power star, HQI-IS 400W, Berlin, FRG). The incident PPFD was varied by placing the lamp at different distances from algal samples. Control tubes were incubated at 38°C in dim light of about 5 μmol m^{-2} s^{-1}. All samples were bubbled with air in order to obtain uniform effect of light treatment for all cells in the suspension. After the photoinhibitory treatment, samples were once again supplied with inorganic carbon, centrifuged, and resuspended in an appropriate volume of medium for the following measurements. Where indicated, the translation inhibitor streptomycin was used before or immediately after the light treatment to a final concentration of 250 μg/mL. Reactivation of photosynthesis after photoinhibition was done at 38°C in dim light of a PPFD of 20 μmol m^{-2} s^{-1}.

Photosynthetic Measurements

Photosynthesis was measured with a Clark-type O_2 electrode (Hansatech, Ltd., Norfolk, UK) at a nonsaturating PPFD of 55 μmol m^{-2} s^{-1} and at 38°C. After the photoinhibitory treatment algal suspensions were centrifuged and resuspended in fresh medium to a final Chl concentration of 5 μg/mL. Two milliliter aliquots of these suspensions (10 μg of total Chl) were transferred to the electrode and bubbled with N_2 to avoid O_2 saturation during the measurements. Prior to the measurements, 20 μL of 1 M $NaHCO_3$ solution was added to certify saturating CO_2 conditions. The light source was an Atlas projector lamp (A1, 215 24V/150W). The rates of photosynthesis were expressed as μmol O_2 evolved mg Chl^{-1} h^{-1} on the basis of electrode signals registered on a chart pen recorder.

Fluorescence Measurements

Fluorescence kinetics were measured at 77K using the trifurcated fiberglass centered fluorescence spectrometer described previously (15). All algal samples used for measurements contained 10 μg Chl in a final volume of 50 μL. Low temperature fluorescence emission spectra were recorded at 77K using the same spectrometer. All samples were normalized to contain 5 μg phycocyanin in a final volume of 50 μL. The emission peaks at 653, 685, 698, and 718 nm were ascribed to phycobilisomes, Chl *a*, PSII reaction centers and PSI reaction centers, respectively, according to Fork and Mohanty (5). All results presented here are the average values of at least three independent replicates (each in double repetitions) for separately grown cultures. Where indicated, values represent the mean ± SD (n = 6–15).

RESULTS AND DISCUSSION

Growth and Pigment Accumulation

Because of the unknown growth and pigment characteristics of the *Anacystis nidulans psbA* gene-inactivated mutants R2S2C3 and R2K1, we first characterized basic physiological parameters and made comparisons with the wild type R2. As can be seen in Figure 1A, the growth curves of both mutants and the wild type were basically very similar. *A. nidulans* wild-type strain R2 and the mutants R2S2C3 and R2K1 had the same generation time of 8 ± 1 h (Fig. 1A). After 50 h of

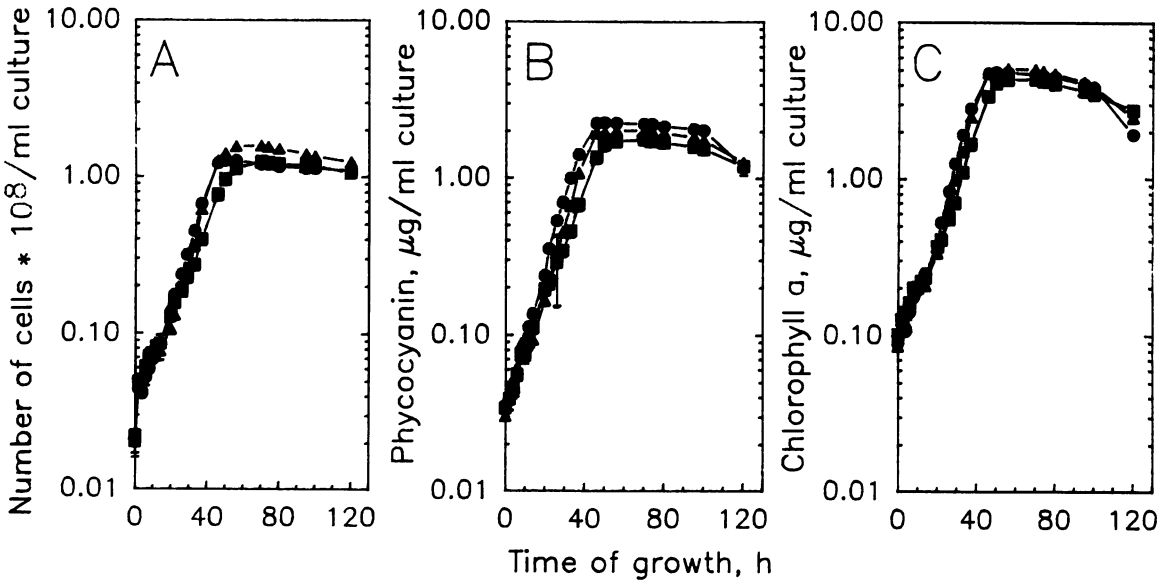

Figure 1. Growth (A), phycocyanin (B), and Chl *a* (C) accumulation of *A. nidulans* wild-type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■).

exponential growth all strains studied rapidly entered a stationary phase (Fig. 1A). Thus, the cultures used for photoinhibitory treatments were taken after approximately 48 h and diluted daily with warm, fresh medium in order to minimize the effects of cell self-shading. The accumulation of phycocyanin and Chl *a* were, like bacterial growth, very similar (Fig. 1, B and C). The phycocyanin to Chl *a* ratios were also similar for the three cultures (Fig. 2). The 48-hr-old cultures used in photoinhibition experiments always had the ratio of the corrected absorptions (A_{625}/A_{678}) in the range of 1.1 to 1.3 which is characteristic for *A. nidulans* growing in well balanced environmental conditions (7). The effect of iron deficiency on the pigment composition of *A. nidulans* documented by Öquist (16, 17) was avoided by a doubling of the iron content. No spectral shift from 679 nm (Chl *a*) toward shorter wavelengths due to low iron level was observed in the cultures.

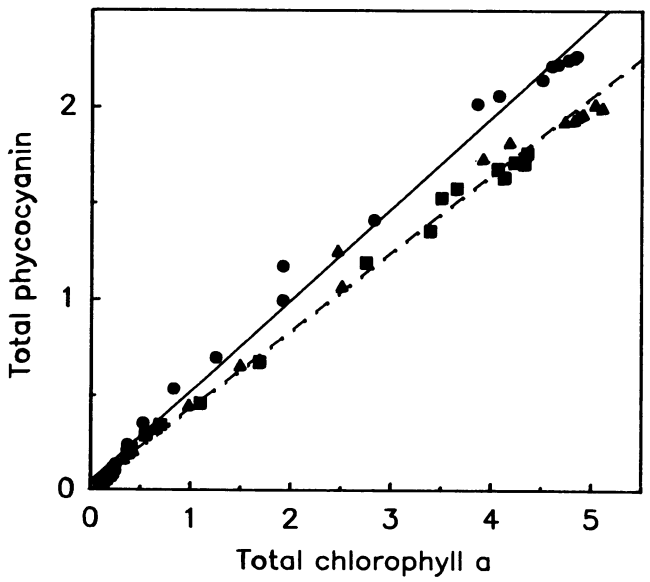

Figure 2. Ratios of phycocyanin to Chl *a* during growth of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■).

Photoinhibition of Photosynthesis

Figure 3A shows that the exposure of *A. nidulans* wild-type R2 and both mutants for 90 min to increasing PPFDs caused inhibition of PSII photosynthetic efficiency as measured by 77K fluorescence kinetics. The R2K1 mutant was more resistant to photoinhibition than the two other strains. However, it took about 10 to 15 min to prepare the samples for fluorescence measurements and meanwhile some recovery might have occurred. Thus, the difference in susceptibility could be due to different abilities to recover during this time. To test this possibility the translation inhibitor streptomycin was added to a final concentration of 250 μg/mL immediately upon termination of photoinhibition. The addition of streptomycin inhibits the recovery process (20, 21) and as can be seen in Figure 3B the three strains were now similar with respect to susceptibility to photoinhibition. However, the R2K1 mutant was still slightly more resistant to photoinhibition than were the other mutant R2S2C3 and the wild type R2 (Fig. 3B).

O_2 evolution measurements at a light limiting PPFD of 55 μmol m^{-2} s^{-1} with the addition of streptomycin after inhibitory light treatment support the 77K fluorescence kinetics data, with the mutant R2K1 being somewhat less sensitive to photoinhibition than the other two cultures (Fig. 4). It should be noted that very good correlation was observed between the photoinhibition measured as the reaction center efficiency by 77K fluorescence kinetics and measured as photochemical activity by O_2 evolution. The correlation coefficients r^2 calculated for all three strains were 0.88 for the wild-type R2, 0.93 for the mutant R2S2C3, and 0.95 for the mutant R2K1.

To investigate whether there was any molecular modifica-

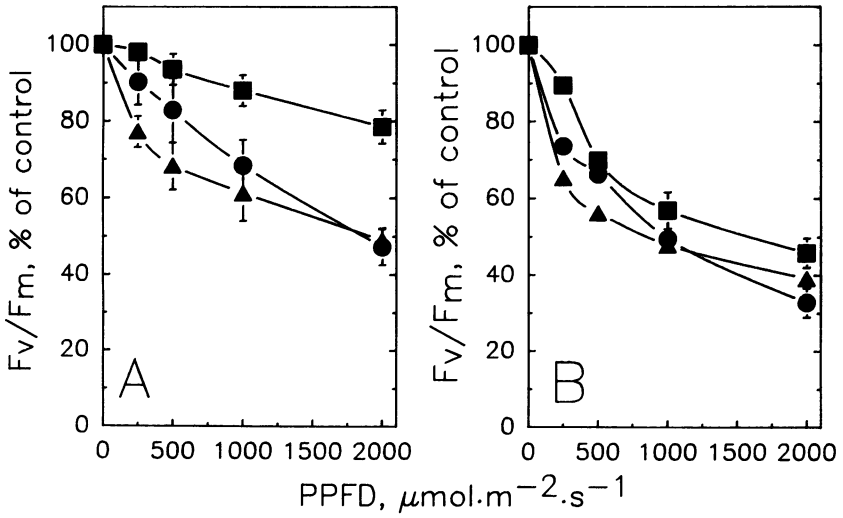

Figure 3. Photoinhibition of PSII photosynthetic efficiency of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■) as measured by 77K fluorescence kinetics. A, Without streptomycin; B, with streptomycin added immediately after photoinhibitory treatment (final concentration 250 μg/mL).

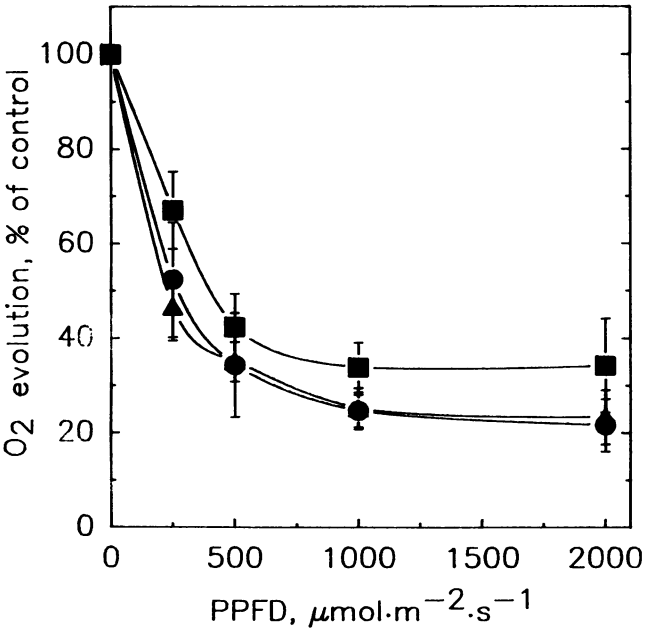

Figure 4. Photoinhibition of O_2 evolution activity of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■). Streptomycin (final concentration 250 μg/mL) added after photoinhibitory treatment.

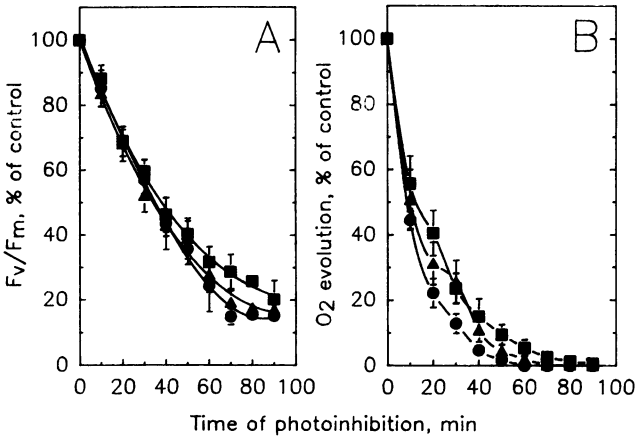

Figure 5. Photoinhibition of photosynthesis of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■) at a PPFD of 250 μmol m^{-2} s^{-1} in samples containing 250 μg/mL of streptomycin. A, 77K fluorescence kinetics; B, O_2 evolution.

tion of the PSII reaction center of the R2K1 mutant that made it more resistant intrinsically to photoinhibition we performed photoinhibition experiments at PPFD of 250 μmol m^{-2} s^{-1} and in the presence of streptomycin (final concentration 250 μg/mL) to inhibit all recovery from photoinhibition. The expected increase of susceptibility to photoinhibition was observed but the three cultures now showed similar sensitivities (Fig. 5, A and B). Thus, the PSII reaction center of the R2K1 mutant *per se* was not more resistant to photoinhibition.

To elucidate whether the increased resistance to photoinhibition in the mutant R2K1 could be ascribed to decreased size of functional antenna because of a detachment of phycocyanin from Chl, analyses of the 77K fluorescence emission spectra at 653 nm were performed. Figure 6 shows that the phycocyanin emission at 653 nm remained unchanged during the photoinhibitory treatment at high PPFDs in the R2K1 mutant as well as in the mutant R2S2C3 and the wild type. Furthermore, the lower F653 emission of the mutants than of wild type contradict a decreased efficiency of energy transfer from phycocyanin to Chl. A detachment of phycocyanin as the reason for increased resistance to photoinhibition of the mutant R2K1 can therefore be excluded.

Recovery from Photoinhibition

The observation that all three cultures showed similar resistance to photoinhibition of photosynthesis in the presence of the translation inhibitor streptomycin constrained us to test the hypothesis that different capacities of the recovery process determine the different susceptibilities to photoinhibition between the R2K1 mutant and the other two strains. This was achieved by exposing cells to PPFD of 500 μmol m^{-2} s^{-1} (fluorescence kinetics measurements) or 250 μmol

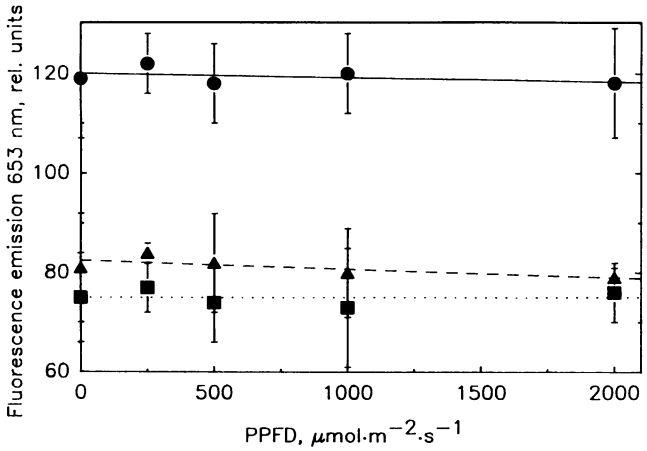

Figure 6. Effect of high PPFDs on the antenna integrity as measured by 77K fluorescence emission at 653 nm of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■).

m^{-2} s^{-1} (O_2 evolution analyses) for 90 min in order to obtain 40 to 50% of photoinhibition. After that, cells were transferred to dim light of 20 μmol m^{-2} s^{-1} without streptomycin being added and the recovery of PSII reaction center efficiency and O_2 evolution were measured. Figure 7, A and B, shows that both in the case of fluorescence kinetics and O_2 evolution measurements the R2K1 mutant recovered approximately 3 times faster from photoinhibition than did the other mutant R2S2C3 and the wild-type R2. The final values of F_v/F_m ratios and O_2 evolution were also higher for the R2K1 mutant after termination of the reactivation process (Fig. 7, A and B).

The results presented in this paper show that the response of the photosynthetic function of *A. nidulans psbA* gene-inactivated mutant, R2S2C3, to excessive light is similar to that of the wild-type R2. However, the R2K1 mutant is more resistant to photoinhibition than both the mutant R2S2C3 and the wild type. Based on the hypothesis that observed photoinhibition of photosynthesis is determined by the balance between photodamage and repair of PSII, we suggest that the major reason to the increased resistance to photoinhibition of the mutant R2K1 is determined by its very high ability to recover from photodamage.

It is well known that the reactivation from photoinhibition depends on the synthesis of the D1 protein, which is the major target of the action of excessive light (1, 8, 9, 24). Thus, the protein resynthesis is a key point in the recovery from photoinhibition. The data presented here strongly indicate that the R2K1 mutant is less susceptible to photoinhibition because of a high rate of recovery, approximately 3 times faster than the mutant R2S2C3 and the wild type. A similar high rate of reactivation was also observed in our earlier experiments on high light grown cells of *A. nidulans* strain 625 (21). Because the *psbAII* and *psbAIII* genes present in the R2K1 mutant are highly expressed under very high light, it would be very interesting to evaluate whether there exists an environmental adaptation mechanism regulating the response of *A. nidulans* to extreme light conditions by 'switching' the *psbA* genes from low and normal light-expressed (*psbAI*) to high light-expressed (*psbAII*, *psbAIII*).

A high stability of the level of the D1 transcript has been shown earlier (11). It indicates that this protein is mainly regulated at the translational level. However, the higher level of the *psbA* gene transcript in high light grown *A. nidulans* compared to low light grown cells as reported earlier (11) suggests a possibility of a long-term acclimation at the transcriptional level. Moreover, Schaefer and Golden (22) have recently shown that there exists an increased proportion of form II (product of *psbAII/III* genes) to form I (*psbAI* gene product) of the D1 protein in thylakoid membranes of wild type of *A. nidulans* grown under high light conditions.

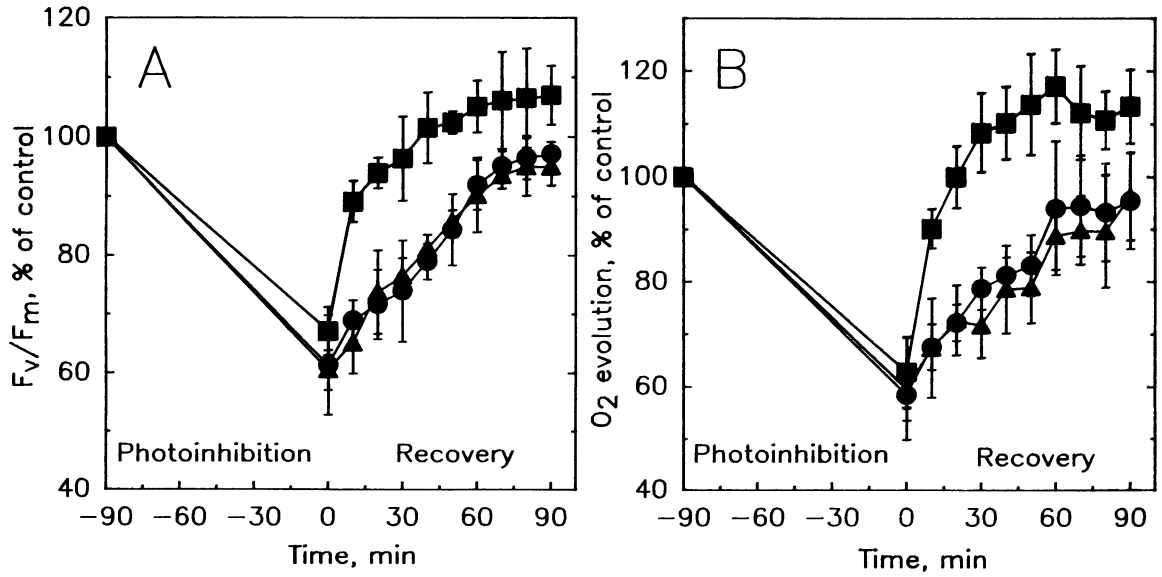

Figure 7. Recovery of PSII photosynthetic efficiency as measured by 77K fluorescence kinetics (A) and O_2 evolution (B) of *A. nidulans* wild type R2 (●) and mutants R2S2C3 (▲) and R2K1 (■). Cells were photoinhibited for 90 min at a PPFD of 500 μmol m^{-2} s^{-1} and recovered during 90 min in dim light of a PPFD of 20 μmol m^{-2} s^{-1}.

In summary, our results suggest that the susceptibility to photoinhibition is regulated by modifying repair (*i.e.* turnover of the D1 protein) rather than modifying the PSII reaction centre *per se.*

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