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Assessment of Photosynthetic Performance of *Prochloron* in *Lissoclinum patella* in hospite by Chlorophyll Fluorescence Measurements

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Two new PAM fluorometers (pulse amplitude modulated) were used in an investigation of photosynthetic performance of *Prochloron* resident as a symbiont in the ascidian *Lissoclinum patella*, growing in a coral reef of Heron Island on the Great Barrier Reef. With a new DIVING-PAM in situ measurements of effective PSII quantum yield ($\Delta F/Fm'$) as a function of quantum flux density (rapid light curves) were carried out in 2.5 m depth in the reef and in a seawater tank. Photosynthetic electron transport rates were measured on in hospite *Prochloron* both in situ and in collected material. Both light-limited and light-saturated yields were exceptionally high. Maximal yields (Fv/Fm) were ~0.83. A new TEACHING-PAM was employed for analysing dark-light induction and light-dark relaxation kinetics in collected samples with *Prochloron* in hospite. Considerable variability in kinetic responses was observed which was found to be at least in part due to differences in O_2 concentration. It is suggested that endogenous reductants feed electrons into the intersystem transport chain, which normally is reoxidized by O_2 (chlororespiration), and that in the dark, the reduction level of PSII acceptors is increased due to a decline in O_2 concentration. The pattern of fluorescence responses differed markedly from those found in cyanobacteria and provides new insights into light-harvesting responses of a photosynthetic prokaryote with a membrane bound light-harvesting system, as contrasted with an extrinsic light-harvesting system.

Key words: Chlorophyll fluorescence — Chlororespiration — Coral reef — PAM fluorometer — Photosynthesis — *Prochloron*.

Since its discovery by Lewin and Withers (1975) *Prochloron* has attracted particular interest in the scientific community, because of the possibility that an ancestral prochlorophyte may have played a crucial role in the evolution of green plant chloroplasts (Lewin 1981, Seewaldt and Stackebrandt 1982, van Valen 1982). *Prochloron* represents an oxygenic photosynthetic prokaryote, which like cyanobacteria contains chlorophyll *a*. However, unlike cyanobacteria it does not contain phycobiliproteins as antenna pigments, but chlorophyll *b*, as in green plants (green algae and higher plants). Hence, in accordance with the endosymbiosis-hypothesis, the first unicellular green algae could have originated from endocytosis of a *Prochloron* cell with a heterotrophic protozoan cell. Analogously, red algae could have originated from endosymbiosis of a cyanobacterium cell within a protozoan host cell. Since the discovery of *Prochloron*, two other prochlorophytes have been found—*Prochlorothrix* (Burger-Wiersma et al. 1986) and *Prochlorococcus* (Chisholm et al. 1988). Molecular phylogenetic studies of these prochlorophytes has indicated that they are a diverse group closely allied to the cyanobacteria (see eg. La Roche et al. 1996) and their proposed ancestry of green chloroplasts has been questioned by Hess et al. (1996), who discovered phycoerythrin in *Prochlorococcus* cells and raised the question of a common ancestor of cyanobacteria and prochlorophytes. If this is the case, then chlorophyll *b* arose independently in prochlorophytes, where it is attached to a light-harvesting protein different from that of green plants (La Roche et al. 1996).

Despite the importance of prochlorophytes in aiding our understanding of plant evolution and the role of chlorophyll *b*, there is little information on the photosynthetic performance of prochlorophytes and *Prochloron* in particular (for early reports, see Withers et al. 1978, Kremer et al. 1982, Critchley and Andrews 1984; for *Prochlorothrix*, see Post et al. 1993; for *Prochlorococcus* see Moore et al. 1995). While their recent discovery may explain much of the lack of detailed information, in the case of *Prochloron* there are the additional difficulties that *Prochloron* exists exclusively as a symbiont in association with didemnid ascidians found only on coral reefs and that so far it has not been possible to culture *Prochloron* cells, which do not survive for long periods of time when isolated from their hosts.

Recently chlorophyll fluorescence quenching analysis has been applied with considerable success for non-invasive assessment of photosynthetic performance of terrestrial plants in their natural environment (for reviews see Krause and Weis 1991, Schreiber et al. 1994). PAM fluorometers

Abbreviations: ETR, electron transport rate; LED, light emitting diode; PAM, pulse amplitude modulation; PAR, photosynthetically active radiation; qN, coefficient of nonphotochemical quenching; qP, coefficient of photochemical quenching; RLC, rapid light curve.

(Pulse-Amplitude-Modulation) can measure chlorophyll fluorescence yield with high selectivity against the background of ambient daylight (Schreiber 1986) and allow the application of saturating light pulses for transient saturation of primary energy conversion at PSII reaction centres. In this way, photochemical and nonphotochemical quenching coefficients can be determined (Schreiber et al. 1986) and the effective quantum yield of energy conversion in PSII can be measured (Genty et al. 1989).

This communication reports on the first measurements of photosynthetic performance of *Prochloron* in hospite in the host *Lissoclinum patella* both in situ and in collected material. It is shown that *Prochloron* displays exceptionally high quantum yields, both under light-limiting and light-saturated conditions and a number of characteristics similar to green chloroplasts.

Materials and Methods

Prochloron was studied as a phycobiont in *Lissoclinum patella* collected in the 'Blue Pools' area of the Heron Island reef east of Gladstone in the Great Barrier Reef (Australia) (see Larkum et al. 1994). The in situ measurements were carried out at 2.5 m depth on the outer reef slope together with the green filamentous alga *Chlorodesmis fastigiata* on a flat rock, well exposed to incident sunlight. Rapid light curves were measured at noon, when the incident quantum flux density was 800 μmol $m^{-2} s^{-1}$ PAR. During measurement of the rapid light curves the samples were darkened by the fibre probe, such that only the actinic light generated by the DIVING-PAM Fluorometer was effective.

Collected samples of *Lissoclinum patella* were kept in seawater tanks with permanent flow-through of fresh seawater in shaded day light (50–150 μmol quanta $m^{-2} s^{-1}$ PAR).

Chlorophyll fluorescence in situ was measured with a new underwater PAM Fluorometer (DIVING-PAM, Walz, Germany). A MINI-PAM Fluorometer (Walz) was employed for measurement of the light response of collected material. Both fluorometers employ 3 μs pulses of a light-emitting-diode (LED) with peak emission at 650 nm as measuring light. Fluorescence is detected at wavelengths above 710 nm. Heat-filtered white light from a halogen lamp serves for actinic illumination and saturation pulses. Saturation pulse intensity was 8,000 μmol quanta $m^{-2} s^{-1}$ PAR and the pulse width was 800 ms. With the help of the saturation pulses the relative quantum yield of photochemical energy conversion in PSII reaction centres can be determined on the basis of two consecutive measurements of relative fluorescence yield (Schreiber et al. 1986, Genty et al. 1989). The first measurement is done in the dark adapted state or any given light state, providing the fluorescence yields Fo or F, respectively [for nomenclature see van Kooten and Snel (1990) and Fig. 4C]. The second measurement is carried out during a saturating light pulse applied in the given state, providing the maximal fluorescence yield, Fm, in the case of a dark-adapted sample, or Fm′ in an illuminated sample. The saturation pulse induced fluorescence increase, ΔF, in relation to maximal fluorescence yield, Fm or Fm′, has been shown to be a good relative measure of quantum yield (Genty et al. 1989):
$\Delta F/Fm = Fv/Fm$ = maximal potential PSII quantum yield of dark-adapted sample,
$\Delta F/Fm'$ = effective PSII quantum yield of illuminated sample, which is lowered with respect to $\Delta F/Fm$ by partial closure of reaction centres and a relative increase of nonradiative energy dissipation.

In view of the fact that electrons leading to CO_2-reduction originate from water splitting in PSII, the overall photosynthetic transport rate (ETR) may be estimated from effective PSII quantum yield:
$ETR = \Delta F/Fm' \times c \times PAR$ μmol electrons $m^{-2} s^{-1}$,
where PAR is the quantum flux density of photosynthetically active radiation. The coefficient c amounts to a value of 0.42 in green leaves (see e.g. Schreiber et al. 1994). It is determined by absorbance of incident light (average 0.84 in leaves) and the assumption that 50% of the absorbed quanta are distributed to PSII. Without knowledge of the true fraction of incident quanta reaching PSII, the same factor of 0.42 as in leaves was also assumed for *Prochloron* resident in *Lissoclinum*.

Rapid light curves (RLC) were measured following a software controlled protocol with 10 s illumination times and intensities increasing in 8 steps. Flexible fibreoptics were used for sample illumination and fluorescence collection. The free end of the fibreoptics was mounted in a 3 × 4 cm black plexiglas holder which during the measurements was gently pressed against the sample, thus darkening the sample and assuring a fixed distance (5 mm) between fibreoptics exit plane and sample. Between sample darkening and RLC-recording only ca. 30 s elapsed, thus assuring that the measured quantum yields at the various quantum flux densities were characteristic for the light adaptation state acquired by the phycobionts in their natural environment.

For kinetic recordings a prototype of the newly developed TEACHING-PAM Fluorometer (Walz) was used in conjunction with a laptop personal computer. This fluorometer employs a 650 nm LED for pulse modulated measuring light, a 660 nm LED for actinic light and saturation pulses, and a 730 nm LED for selective excitation of PSI. Saturation pulse intensity was 3,500 μmol $m^{-2} s^{-1}$ PAR, which was sufficiently high to reach maximum fluorescence yield (Fm-plateau) within the 800 ms pulse length. The light from the three LEDs is collected by a miniature perspex cone with 8 mm entrance and 2 mm exit diameters. This cone at the same time serves the purpose of collecting fluorescence from the sample and guiding it to the photodiode detector. Samples were cut to approx. 8 × 8 mm size, with the fibrous, jelly-like coat being removed by a razor blade. They were placed with the *Prochloron* containing layer on the bottom window of a small cuvette filled with fresh seawater, such that only a 0.1 mm thick glass window separated the *Prochloron*-carrying layer from the exit plane of the measuring head. Temperature was approximately 25°C. The coefficients of photochemical quenching, qP, and of nonphotochemical quenching, qN, were determined by saturation pulse quenching analysis (Schreiber et al. 1986, 1994; see van Kooten and Snel 1990 for nomenclature). Such quenching analysis involves measurements of minimal and maximal fluorescence yields of the dark-adapted sample (Fo and Fm, respectively), and the measurements of momentary and maximal yield in a given light state (F and Fm′, respectively). The quenching coefficients were automatically calculated on the basis of the following equations:

$$qP = \frac{Fm' - F}{Fm' - Fo} \qquad qN = \frac{Fm - Fm'}{Fm - Fo}$$

Results and Discussion

Rapid light response curves—The photosynthetic performance of *Prochloron* resident in *Lissoclinum patella*, a colonial ascidian growing at 2.5 m depth on the reef, was

assessed in situ (see Materials and Methods). Fig. 1 shows plots of effective quantum yield and of relative electron transport rate versus the flux density of incident photosynthetically active radiation. The data were sampled automatically by running a so-called "rapid light curve", a method which has been recently introduced for rapid assessment of the photosynthetic capacity and light adaptation state of plants in their natural environment (Critchley and Gademann, in preparation). Although the illumination periods of 10 s at each intensity may be too short to reach true steady-state, these measurements give a good relative evaluation of the photosynthetic performance. The sample had been exposed to ca. 800 μmol quanta $m^{-2} s^{-1}$ PAR of sunlight at midday, before it was covered by the fiberoptics adaptor (see Materials and Methods) and the "rapid light curve" recording was started immediately thereafter. When light intensity was gradually increased from darkness to 1,200 μmol quanta $m^{-2} s^{-1}$ PAR, the effective quantum yield $\Delta F/Fm'$ (Genty et al. 1989) displayed a relatively small decrease from 0.52 to 0.35 (Fig. 1A). Since the flux of photosynthetically active radiation in the exit plane of the fiberoptics was known, it was possible to derive an estimate of the relative rate of charge separation at PSII, which at a first approximation is directly related to the electron transport rate, ETR (Genty et al. 1989). From Fig. 1B it is apparent, that ETR was not saturated at 1,200 μmol quanta $m^{-2} s^{-1}$ PAR. For comparison, Fig. 2 shows the corresponding responses of the green macroalga *Chlorodesmis festigiata*, growing in close proximity to the investigated *Lissoclinum* sample. It may be noted that the photosynthetic capacity of *Prochloron* in *Lissoclinum* is distinctly higher than that of *Chlorodesmis*.

Rapid light response curves of *Prochloron* in collected *Lissoclinum* were studied after exposure to shaded light in a seawater tank. An example of such a measurement is given in Fig. 3. It is apparent that, in comparison to the in situ measurement, quantum yields were distinctly higher at low light (Fig. 3A), while approximately the same high ETR values were reached at high intensity. This suggests that the PSII quantum efficiency of *Prochloron* in situ at midday was lowered by a high yield of nonradiative energy dissipation, a phenomenon (midday depression) which has been extensively investigated in higher plant leaves (for a review, see Björkman and Demmig 1994).

Dark-light induction transients of collected samples—*Prochloron* resident in *Lissoclinum patella* maintained high photosynthetic activity in hospite for about 2 days after samples were collected from the reef and kept in sea water tanks in shaded daylight. Fig. 4 shows typical recordings of dark-light induction curves measured with such samples which were transferred from ambient room light

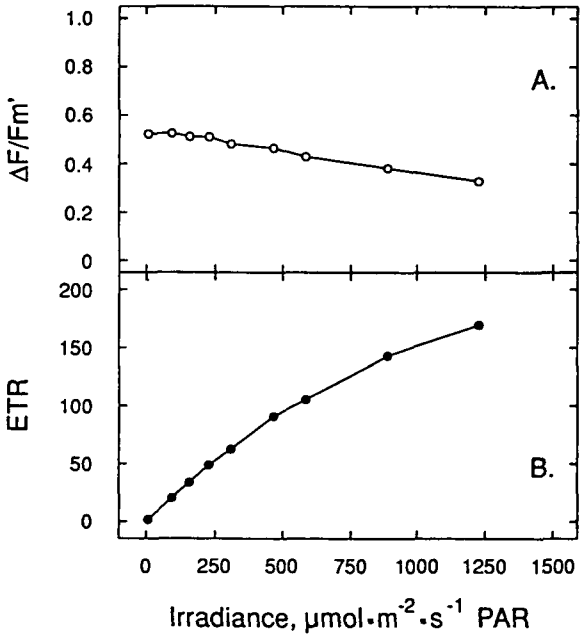

Fig. 1 Effective quantum yield of PSII, $\Delta F/Fm'$, and relative electron transport rate, ETR, of *Prochloron* in *Lissoclinum patella* assessed in situ at 2.5 m depth as function of quantum flux density. Measurement by automized "rapid light curve" using the DIVING-PAM Fluorometer, with 10 s illumination at each intensity setting. The irradiance values are corrected for attenuation by a 5 mm jelly-like coat covering the ascidian. ETR is calculated according to the formula $ETR = \Delta F/Fm' \times 0.42 \times PAR$ μmol electrons $m^{-2} s^{-1}$. See text for further information.

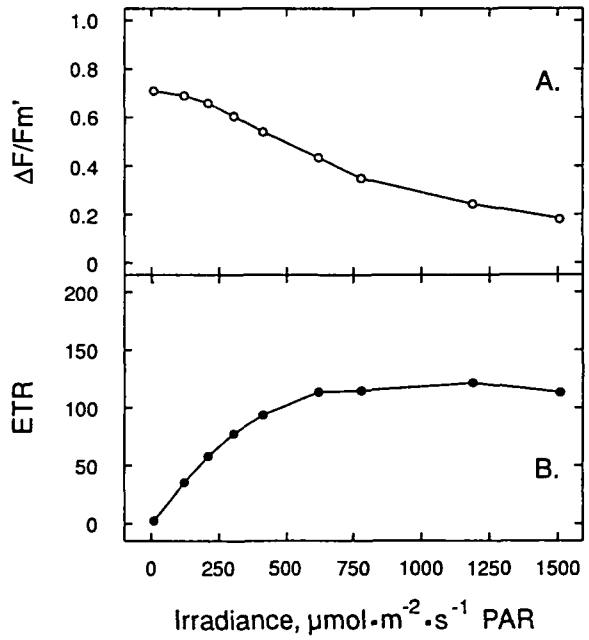

Fig. 2 Effective quantum yield of PSII, $\Delta F/Fm'$, and relative electron transport rate, ETR, of *Chlorodesmis festigiata* assessed in situ at 2.5 m depth as function of quantum flux density. See legend to Fig. 1 and text for further details.

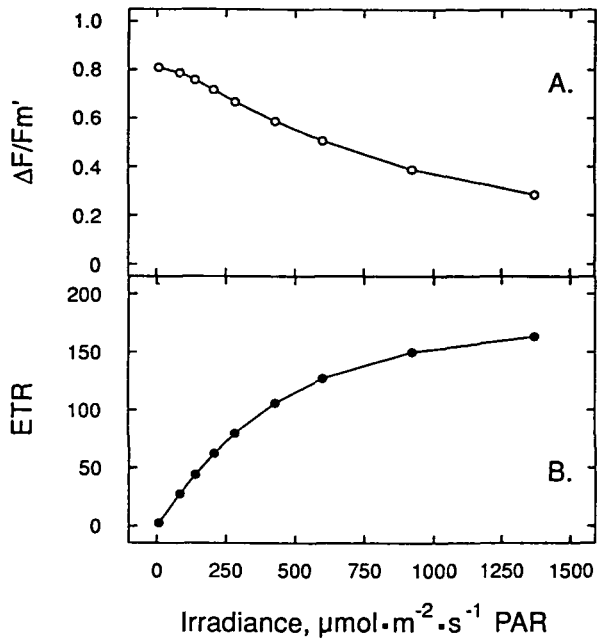

Fig. 3 Effective quantum yield of PSII, $\Delta F/Fm'$, and relative electron transport rate, ETR, of *Prochloron* in a collected sample of *Lissoclinum patella* maintained in a seawater tank as function of quantum flux density. Measurement by automized "rapid light curve" using MINI-PAM Fluorometer, with 10 s illumination at each intensity setting. The sample had been exposed to ca. 80 μmol m^{-2} s^{-1} PAR before start of the measurement. See legend to Fig. 1 and text for further details.

to the measuring head only briefly (ca. 1 min) before start of the experiment. Saturating light pulses were applied repetitively every 20 s in order to assess photochemical and nonphotochemical quenching coefficients, qP and qN, and to determine effective quantum yield, $\Delta F/Fm'$. Before each recording (~30 s), the maximal quantum yield, Fv/Fm, was determined by application of a saturation pulse. Comparison of the four curves displayed in Fig. 4A–C reveals considerable differences in the induction kinetics of the fluorescence yield parameters F and Fm', the effective quantum yield, $\Delta F/Fm'$, and the quenching coefficients, qP and qN. There are also substantial differences in the quasi-steady-state values reached during the 5 min illumination periods. For comparison, the various parameters of the four samples are summarised in Table 1. While the cause for the largely differing responses presently is unknown, the following characteristic features may be pointed out:

(1) In all samples the maximal quantum yield, Fv/Fm, is quite high, indicating high PSII activity.

(2) In all samples shortly after onset of actinic illumination (~1 s), the effective quantum yield, $\Delta F/Fm'$, and qP are relatively low, indicating dark-inactivation of photosynthetic electron transport. Hence, the four samples differ mainly in their capability to reactivate electron transport during illumination.

(3) Except for the least active sample D, the induction kinetics of *Prochloron* are characterized by close to parallel increases of $\Delta F/Fm'$, qP and qN.

(4) In agreement with point (3), the samples with the highest steady-state yields, $\Delta F/Fm'$, also display the

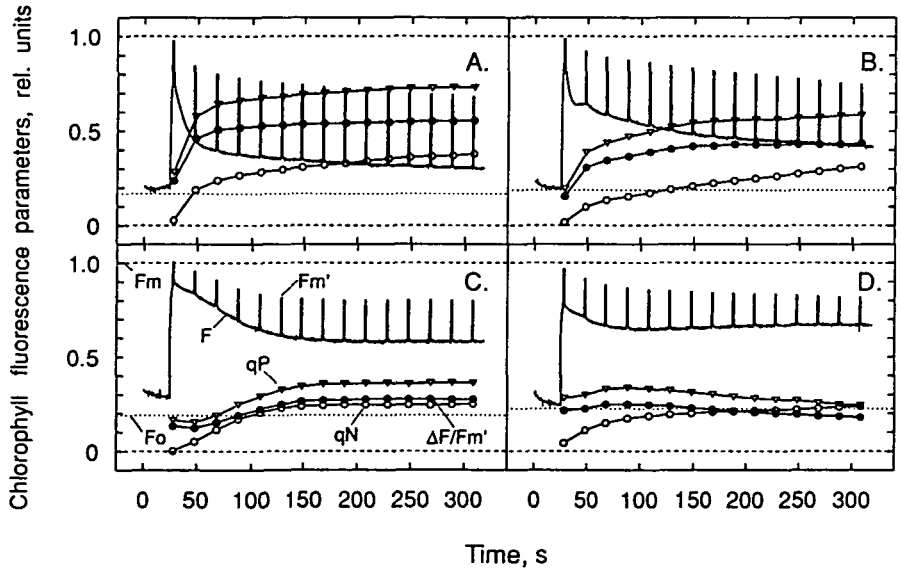

Fig. 4 Recordings of dark-light induction curves with fluorescence quenching analysis by the saturation pulse method of 4 different samples of *Prochloron* in *Lissoclinum patella* using the TEACHING-PAM Chlorophyll Fluorometer. The notations of the characteristic fluorescence levels and of on-line calculated fluorescence parameters are given in panel C. Actinic light intensity, 170 μmol quanta m^{-2} s^{-1}; peak-wavelength 660 nm. See text for further details.

Table 1 Comparison of characteristic chlorophyll fluorescence parameters observed with four different samples of *Lissoclinum patella* containing *Prochloron*

Sample	Before illumination	1 s illuminated		5 min illuminated	
	Fv/Fm	ΔF/Fm'	qP	ΔF/Fm'	qP
A	0.831	0.235	0.283	0.551	0.730
B	0.812	0.158	0.195	0.438	0.585
C	0.810	0.133	0.164	0.274	0.360
D	0.777	0.215	0.279	0.178	0.244

Samples A–D were identical to the samples used in the kinetic recordings of Fig. 4A–D.

highest qP and qN values.

Light-dark relaxation kinetics—Additional information supplementing the information from dark-light induction curves, may be obtained by recording light-dark relaxation kinetics of the characteristic fluorescence parameters. A typical recording is shown in Fig. 5A. This recording was preceded by a 5 min illumination period, during which an induction curve was measured (not shown) which closely resembled that of Fig. 4B. The observed light-dark relaxation kinetics of *Prochloron* in hospite differ from the well studied characteristics of green leaves (see e.g. Quick and Stitt 1989) in two major respects:

(1) Only a small part of nonphotochemical quenching, qN, relaxes within the first minute of darkness.

(2) Relaxation of another part of qN is closely followed by a surprising increase of fluorescence yield, F, concomitant with a decrease of ΔF/Fm'.

For an interpretation of these observations the results of Fig. 5B are relevant, which were obtained from an experiment similar to that of Fig. 5A, except that simultaneously with the light-dark transition, far-red light was turned on. It may be noted that the far-red counteracted the rise of F and the decrease of ΔF/Fm', without affecting the two-step decrease of qN. These observations may be explained by the following hypothesis:

Part of energy-dependent nonphotochemical quenching in *Prochloron* can be maintained in the dark by chlororespiratory electron transport, which involves electron donation from reduced cytoplasmic components to the intersystem electron transport chain, possibly via an intersystem electron carrier, which could be a component of the cyt *b/f* complex. As the diffusion of molecular O_2 from the surrounding water to the *Prochloron* cells in hospite is severely restricted, the O_2-concentration within the cells may drop in the dark to a level which does not support chlororespiration any more, such that the qN associated with this type of electron flow will decline. With weak far-red light, which selectively drives PSI, it is possible to prevent excessive dark-reduction of the plastoquinone-pool at low O_2-concentration. This prevents the accumulation of reduced primary PSII acceptor, Q_A, and therefore the drop in PSII quantum yield, ΔF/Fm'.

Rapid induction kinetics—Fig. 6 shows recordings of rapid induction kinetics of *Prochloron* in hospite after different dark-adaptation times. With increasing dark-time (1–10 min) the following changes are observed:

(1) The dark fluorescence yield, Fo, is increased.

(2) The initial fluorescence rise upon onset of actinic illumination (O-I transient) is accelerated.

(3) A pronounced dip (I-D transient) is developed.

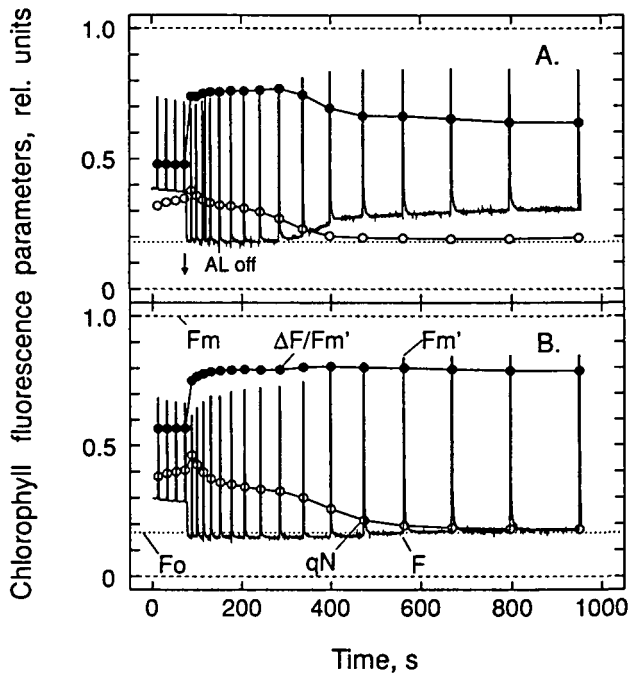

Fig. 5 Light-dark relaxation kinetics of fluorescence yield and on-line calculated fluorescence parameters of *Prochloron* in *Lissoclinum patella*. Actinic light of 170 μmol quanta m^{-2} s^{-1} was turned off were indicated (AL off). A. Following AL off, the sample was fully darkened. B. Simultaneously with AL off, continuous far-red light at an intensity of 10 W m^{-2} was applied. The notations of the characteristic fluorescence levels and of on-line calculated fluorescence parameters are given in panel B. Two different samples were used in the experiments of Fig. 5A and B.

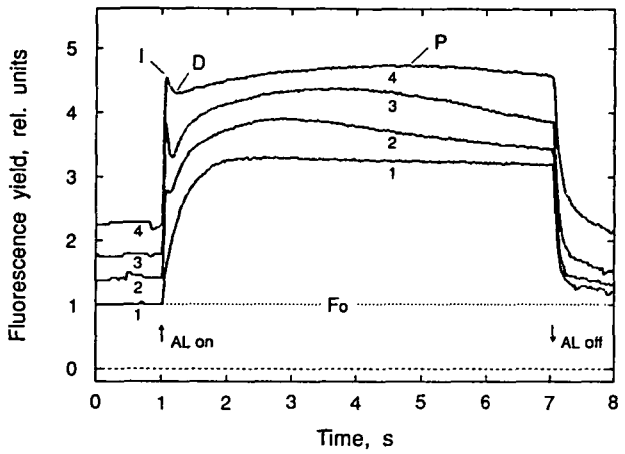

Fig. 6 Rapid dark-light induction transients of *Prochloron* in *Lissoclinum patella* following different dark-light adaptation times. Samples were transferred from ambient room light on the measuring head of the TEACHING-PAM and induction curves measured after 1 min (curve 1), 3 min (curve 2), 6 min (curve 3) and 10 min (curve 4) dark adaptation. The characteristic fluorescence levels are indicated. Actinic light (AL) intensity, 40 μmol quanta $m^{-2} s^{-1}$ PAR.

(4) The amplitude of the peak (P) is increased.

Very similar phenomenology has been previously observed with increasing times of dark anaerobic incubation of the green unicellular alga *Scenedesmus obliquus* (Schreiber and Vidaver 1974). These results also support the hypothesis (see above) that in the dark *Prochloron* in hospite tends to become anaerobic and that some endogenous reductant feeds electrons into the photosynthetic intersystem chain at the acceptor side of PSII.

Concluding Remarks

It may be concluded that chlorophyll fluorescence measurements can provide important information on the photosynthetic performance of *Prochloron* in hospite. The experiments with the DIVING-PAM have allowed a detailed study of this extraordinary symbiont in hospite in its natural environment where it is obviously performing exceptionally well. When *Prochloron*, together with its host *Lissoclinum patella* was removed from the reef and kept in a sea water tank, it kept high maximal quantum yield, Fv/Fm, for 1–2 days, but during this time its effective quantum yield in continuous light, $\Delta F/Fm'$, gradually declined. Experiments with collected material showed considerable variability in dark-light induction transients, the cause of which remains to be clarified by future work. One parameter which emerged as particularly important is O_2 concentration. The data are in agreement with the hypothesis that in *Prochloron*, just as in various green algae and cyanobacteria (see e.g. Mi et al. 1992), endogenous reductants feed electrons into the intersystem electron transport chain, which normally is reoxidised by molecular oxygen. This 'chlororespiration' (Bennoun 1982) may maintain an ATP-generating ΔpH in the dark. Hence, here it may play an important role for the survival of the phycobiont in the dark. At present it is not clear at what site the electrons enter the chain, whether an NAD(P)H-dehydrogenase is involved, where the electrons are transferred to O_2 and whether a specific oxidase is involved. Also there remains the interesting possibility of a transfer of reducing power from the host to the phycobiont. As shown by Critchley and Andrews (1984), *Prochloron* displays extraordinary plasma membrane permeability properties, and rapid exchange of metabolites may be part of the mechanism of this symbiosis. In this regard it should be noted that ascidians possess a range of unique redox systems for controlling Eh and oxygen supply (Taylor et al. 1994) and *Prochloron* in *Lissoclinum patella* has been shown to contain the essential enzymes (superoxide dismutase, ascorbate peroxidase and catalase) protecting against potentially harmful effects of excess oxygen (Lesser and Stochaj 1990). Recently a sensitive microfibre probe for O_2 has been introduced (Klimant et al. 1995) which can be applied in combination with a microfibre chlorophyll fluorescence probe (Schreiber et al. 1996). It is hoped that these two techniques can be deployed, without disturbing the delicate relationship between host and symbiont, to answer these questions.

In relation to the question of phylogeny of chloroplasts of green plants, it may not be fortuitous that *Prochloron* in hospite displays Fv/Fm-values up to 0.83. Such high values to our knowledge so far have been only observed with higher plant leaves (see also Büchel and Wilhelm 1993) and certainly not in cyanobacteria. This calls for a thorough comparative study of fluorescence parameters in cyanobacteria, prochlorophytes and the various groups of micro- and macroalgae. For such studies it will be important to vary the wavelength of excitation for optimal absorption by different pigment systems using for example the Xe-PAM (Schreiber et al. 1993).

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