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Emerging techniques

Physcomitrella patens and *Ceratodon purpureus*, mosses as model organisms in photosynthesis studies

Leeann E. Thornton[1,**], Nir Keren[1,**], Itzhak Ohad[2] & Himadri B. Pakrasi[1,*]
[1]*Department of Biology, Washington University, St. Louis, MO 63130, USA;* [2]*Department of Biological Chemistry and Minerva Center of Photosynthesis Research, The Hebrew University of Jerusalem, Jerusalem 91904, Israel;* **Author for correspondence (e-mail: pakrasi@wustl.edu; fax: +1-314-935-6803);* ***These authors contributed equally to this study*

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Abstract

With the discovery of targeted gene replacement, moss biology has been rapidly advancing over the last 10 years. This study demonstrates the usefulness of moss as a model organism for plant photosynthesis research. The two mosses examined in this study, *Physcomitrella patens* and *Ceratodon purpureus*, are easily cultured through vegetative propagation. Growth tests were conducted to determine carbon sources suitable for maintaining heterotrophic growth while photosynthesis was blocked. Photosynthetic parameters examined in these plants indicated that the photosynthetic activity of *Ceratodon* and *Physcomitrella* is more similar to vascular plants than cyanobacteria or green algae. *Ceratodon* plants grown heterotrophically appeared etiolated in that the plants were taller and plastids did not differentiate thylakoid membranes. After returning to the light, the plants developed green, photosynthetically active chloroplasts. Furthermore, UV-induced mutagenesis was used to show that photosynthesis-deficient mutant *Ceratodon* plants could be obtained. After screening approximately 1000 plants, we obtained a number of mutants, which could be arranged into the following categories: high fluorescence, low fluorescence, fast and slow fluorescence quenching, and fast and slow greening. Our results indicate that *in vivo* biophysical analysis of photosynthetic activity in the mosses can be carried out which makes both mosses useful for photosynthesis studies, and *Ceratodon* best sustains perturbations in photosynthetic activity.

Abbreviations: CCD – Charge-coupled device; DCMU – dichlorophenyl-dimethylurea; PSII – photosystem II; EST – expressed sequence tag

Introduction

Some traits of an ideal organism for photosynthesis studies include photosynthetic machinery that is easily purified and can be analysed *in vivo*, and growth in the absence of photosynthetic activity. Some examples of model organisms used in photosynthesis studies are: spinach, from which large amounts of biochemically active material can be isolated, *Chlamydomonas reinhardtii*, a photosynthetic eukaryote for which biophysical measurements can be made *in vivo*, and *Synechocystis* sp. PCC 6803, the first photosynthetic organism with a completely sequenced genome. *In vivo* activity measurements can be made in the cyanobacterium, *Synechocystis* 6803, and whole genome analysis (Kaneko et al. 1996) has facilitated a molecular

understanding of many photosynthetic proteins through reverse genetics. Highly developed tools for biological studies in *Arabidopsis thaliana* have recently made this plant a useful model for photosynthesis studies. For example, *Arabidopsis* can be germinated on sucrose-containing solid medium for the isolation of photosynthetic-deficient mutants (Meurer et al. 1998; Pesaresi et al. 2003; Walters et al. 2003). Mosses have also been developed as model organisms in land plant studies, such as hormone response, cell polarity, and secondary metabolism. Here we describe a number of attributes of the mosses, *Physcomitrella patens* and *Ceratodon purpureus*, which will make them useful tools in photosynthesis research.

An useful approach for studying protein function is through gene knockouts, where changes that result from the single alteration are examined, and several methods have been developed for this purpose in genetic research. In *Arabidopsis*, T-DNA mutagenesis and RNA inhibition have been used to isolate mutants deficient in a specific gene. However, efficient targeted gene replacement had not been demonstrated in a eukaryotic photosynthetic organism until recently.

Genetic studies in mosses were initially developed as its dominant haploid life stage is a useful tool for plant development analysis (Cove et al. 1991). Subsequently, targeted gene replacement in these plants was discovered with the nuclear gene knockout of a chloroplast division gene, FtsZ, in *Physcomitrella* (Strepp et al. 1998). Other examples of this discovery have subsequently been published (Girod et al. 1999; Imaizumi et al. 2002; Koprivova et al. 2002; Zank et al. 2002). Targeted gene replacement studies in *Ceratodon* have not yet been published. Further contributions to moss genetic studies include the development of a gene trap library (Hiwatashi et al. 2001) and RNAi techniques (Bezanilla et al. 2003), as well as an extensive expressed sequence tag (EST) database for *Physcomitrella* (PhyscoBase, http://moss.nibb.ac.jp/). The 80,000 ESTs in the public database have been configured into >21,000 contigs that have recently been translated into a microarray chip for transcriptome analysis, and complete genome sequencing is underway at the Joint Genome Institute, sponsored by DOE. (http://www.jgi.doe.gov/sequencing/why/CSP2005/physcomitrella.html, 10-18-04). Upon completion of this sequencing project in 2005 (R. S. Quatrano, personal communication), *Physcomitrella* will be the largest completely sequenced plant genome.

Mosses appear to have diverged from vascular plants greater than 400 million years ago, and they provide an evolutionary middle step between free-living cyanobacteria and vascular plants. Analysis of photosynthesis protein phosphorylation events place mosses in an intermediate group between angiosperms and phycobillisome-containing organisms (Pursiheimo et al. 1998). Even though much of the photosynthetic machinery is conserved between plants, algae, and cyanobacteria, some changes have occurred. Whole genome analysis is beginning to decipher the origin and evolution of photosynthesis genes as they passed through several bacterial species to eukaryotes (Raymond et al. 2002). Because photosynthesis is a process conserved between cyanobacteria, algae, mosses and vascular plants, studying it in all of these systems will provide insight into the evolution of land plants.

Mosses are simple plants whose primary life stage consists of filamentous protonemal cells and small leafy shoots, both of which are haploid (Cove et al. 1997). Two mosses examined in this study, *Physcomitrella* and *Ceratodon*, are easily cultured by vegetative propagation. Protoplast transformation has been demonstrated as an efficient method of introducing foreign DNA into both *Physcomitrella* and *Ceratodon* (Schaefer et al. 1991; Zeidler et al. 1999). Examining mutants is facilitated by the predominantly haploid nature of the moss life cycle, which eliminates the need for subsequent generations after mutagenesis. The cells are large (approximately 150×20 μm for *Physcomitrella*, 260×15 μm for *Ceratodon*, data not shown) and transparent, so chloroplasts can be easily visualized. Furthermore, as described previously, thylakoid membranes can be isolated from mosses for *in vitro* analysis of photosynthetic properties (Pursiheimo et al. 1998). Here we examine the two mosses for their potential use in studies on photosynthesis. We show that *in vivo* photosynthetic activity analysis can be done on both organisms, and *Ceratodon* is best able to sustain perturbations in photosynthetic processes.

Materials and methods

Culture conditions

All reagents are from Sigma (St. Louis, Missouri), unless otherwise noted. Growth media (BCD) for *Physcomitrella* and *Ceratodon* were essentially as described previously (Ashton and Cove 1977), with Hoagland's A–Z for trace elements and 0.1 M $MgSO_4$, 0.18 M KH_2PO_4, 1 M KNO_3, 4.5 mM $FeSO_4$ and 1 mM $CaCl_2$. Solid BCD was supplemented with 0.8% high gel strength agar. BCDA medium contained 5 mM di-ammonium (+) tartrate as a growth supplement. Cultures were grown under continuous light (50 μmol photons m^{-2} s^{-1}) at 25 °C. Colonies were inoculated by transferring approximately 2 mm of growing protonemal tissue to a new plate. Growth media was supplemented with 0.5% (w/v) glucose for heterotrophic growth and 10 μM dichlorophenyl-dimethylurea (DCMU) for photosynthesis inhibition. Dark grown plants were wrapped in foil and kept in a dark box under the same growth conditions as the continuous light cultures.

PS II fluorescence

For screening, fluorescence traces from dark-adapted (3 min) mosses were collected using a FluorCam 690M CCD camera-based system (P. S. Instruments, Brno, Czech Republic) integrated with FluorCam software for driving the protocol. Saturating light pulses (0.8 s, 500 μmol photons m^{-2} s^{-1}) and actinic light (100 μmol photons m^{-2} s^{-1}) were used to determine variable fluorescence and maximum fluorescence (F_m). This CCD-based system was used to screen up to 50 mutant plants at one time.

Analytical PSII fluorescence measurements were made on a dual modulation kinetic fluorimeter, FL100 (P. S. Instruments, Brno, Czech Republic) (Nedbal et al. 1999). Protonemal tissue was suspended in tap water in a cuvette, and dark-adapted for 3 min. prior to measurements. For Q_A oxidation kinetics (fluorescence decay), four low voltage, 3 μs, measuring flashes were used to collect the initial fluorescence (F_0) of the centers before a saturating high voltage, 30 μs, actinic flash excited the centers for F_m. After the actinic flash, measuring flashes were used to collect fluorescence information from 1 ms after the actinic flash through 32 s. For fluorescence rise kinetics, a series of alternating 5 μs, 50% voltage, actinic flashes and low voltage, 3 μs measuring flashes were used to excite the centers to F_m over 2 s.

Flash-induced oxygen evolution

Flash-induced oxygen evolution measurements were made on a bare platinum electrode (Artesian Scientific Co., Urbana, Illinois) as described in previous studies (Bricker et al. 2001). Flashes were supplied by an integrated, computer-controlled Xenon flash lamp (20 μs width at 1/2 height). For the measurements of S-state distributions and Kok parameters, protonemal tissue was grown on agar plates as described above. Tissue was applied as a thick mat to the bare platinum electrode, and wetted with buffer (10 mM Tes-KOH, pH 8.0, 100 mM NaCl, 20 mM $NaNO_3$, and trace minerals). A clear plastic disk was placed over the wet tissue to generate salt bridge to the AgCl electrode. The tissue was given a train of 20 saturating flashes at 3 Hz before incubation for 10 min in the dark. The electrode was polarized at 0.73 V for 1 min, and a series of 16 flashes were applied. Data points were collected at 100 μs intervals for the duration of the flash train. The collected data were distilled to 16 maximum values (peaks) corresponding to the flashes using software developed for this purpose (R. Thornton). Peak data for each experiment was fitted to a four-step, homogeneous model for manganese cluster S-state cycling (Meunier 1993). Model-fitting calculations were carried out using MathCad software (MathSoft Engineering and Education, Inc., Cambridge, Massachusetts).

Transmission electron microscopy

Moss filaments were fixed overnight at 4 °C in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, followed by washing and postfixing overnight with 2% osmium tetraoxide in the same buffer. Samples were dehydrated in a series of graded alcohols and embedded in Epon (Luft 1961). Sections (0.5–0.6 μm) were stained with uranyl acetate and lead citrate before imaging (Phillips EM300).

Mutagenesis

Ceratodon tissue grown for 8 days on solid BCDA was used to isolate protoplasts for UV mutagenesis essentially as described previously (Grimsley et al. 1977). After incubating protonemal tissue with Driselase at 25 °C for 25 min, the protoplasts were filtered through sterile 50-μm nylon mesh. Isolated protoplasts were plated on regeneration medium as described previously (Grimsley et al. 1977). The protoplasts were irradiated with 5 W m^{-2} UV light for 4 min, which produced approximately 95% kill. The plates were kept in dark for 2 days to prevent photo-repair, and then moved to normal growth conditions. After 7 days of regeneration, 1456 plants were picked to BDCA-glucose plates for screening.

Results

Photosynthetic activity measurements

Photosynthetic activity of *Physcomitrella* and *Ceratodon* was measured using a non-invasive fluorescence imaging technique. Fluorescence rise (Figure 1a) and decay (Figure 1b) kinetics were measured on *Physcomitrella* and *Ceratodon* filaments grown on solid media. Rise kinetics were measured using continuous light, which gradually activates all of the PS II centers to a steady-state fluorescence. Decay kinetics were measured after application of a light pulse, which creates a maximum charge separation in all centers at the same time and allows the measurement of decreasing fluorescence as the system recombines. These

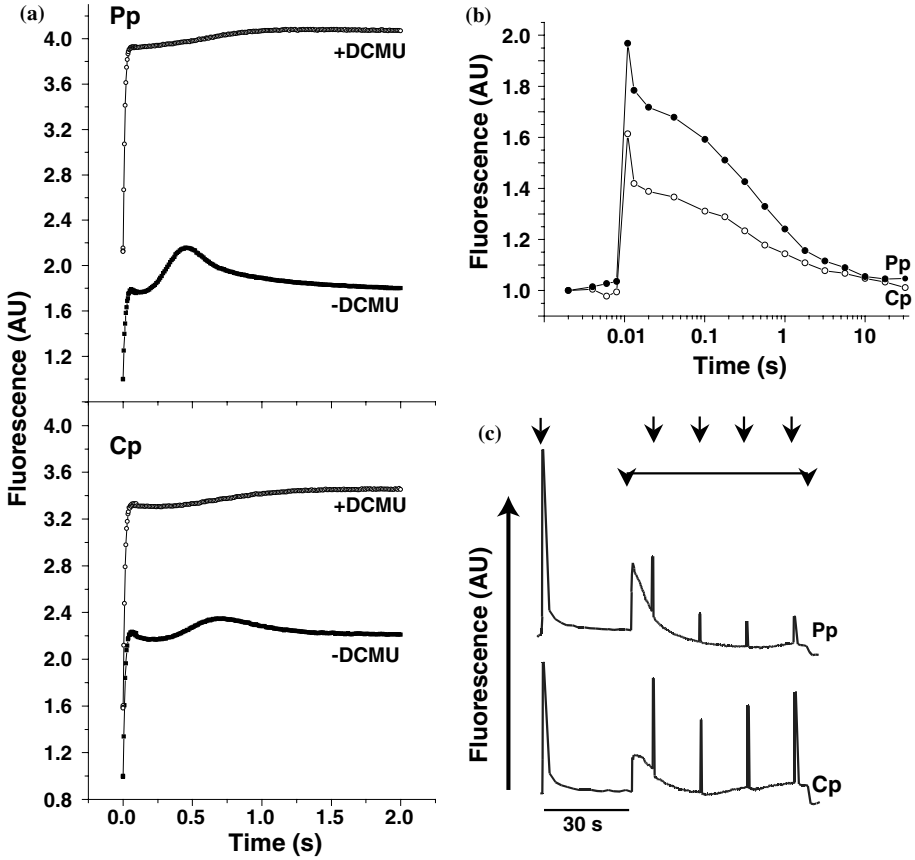

Figure 1. Measurements of photosynthetic activity in mosses. (a) Dual modulation kinetic fluorimeter measurement of rise kinetics of dark-adapted (3 min) *Ceratodon* (Cp) and *Physcomitrella* (Pp). Traces are shown for cells incubated with (+DCMU) and without (−DCMU) 0.2 mM DCMU. F_v/F_m, 0.71 (Cp) and 0.75 (Pp) (b) Dual modulation kinetic fluorimeter measurement of decay kinetics of dark-adapted (3 min) *Ceratodon* (Cp) and *Physcomitrella* (Pp). $t_{1/2}$, 0.112 (Cp) and 0.226 (Pp) (c) Fluorescence traces of dark-adapted (3 min) *Ceratodon* (Cp) and *Physcomitrella* (Pp) using a CCD camera based system. Arrows indicate flashes of saturating light (0.8 s, 500 μmol photons m^{-2} s^{-1}) and the line indicates the duration of actinic light (100 μmol photons m^{-2} s^{-1}).

results show that *in vivo* analytical fluorescence measurements can be obtained from moss tissue without having to extract membranes.

Figure 1c shows fluorescence traces from dark-adapted mosses measured in a CCD camera device suitable for screening up to 50 colonies of plants at one time. Saturating light pulses (arrows) and actinic light (bar with arrow heads) were used to determine F_m. The observed quenching and recovery of F_m throughout the actinic light exposure are typical of most vascular plants, whereas less fluorescence quenching and recovery can be measured in unicellular algae, like *C. reinhardtii* (Keren and Ohad 1998). This procedure demonstrates an efficient method for screening large numbers of photosynthesis-deficient moss mutants.

Flash-induced oxygen evolution was measured in *Physcomitrella* and *Ceratodon* protonemal tissue (Figure 2). The flash pattern for *Physcomitrella* cells is similar to the pattern previously reported for spinach thylakoid membranes (Turcsanyi and Vass 2000), and cyanobacterial cells (Engels et al. 1994), with the highest yields on the third and fourth flashes. This experiment suggests that the PSII centers are in the S_1 and S_0 states after a 10 min-dark incubation, and was verified by fitting the data to a 4-step homogenous model (Meunier 1993) (data not shown). This S-state distribution is not observed for the *Ceratodon* cells, which had a maximum oxygen yield on the fifth flash (Figure 2). The *Ceratodon* data cannot be fitted to a 4-step model, and longer dark incubation time (30 min) did not change the observed pattern (data not shown). This interesting result suggests that the *in vivo* PS II oscillation pattern in *Ceratodon* is different from *Physcomitrella* and should be examined further.

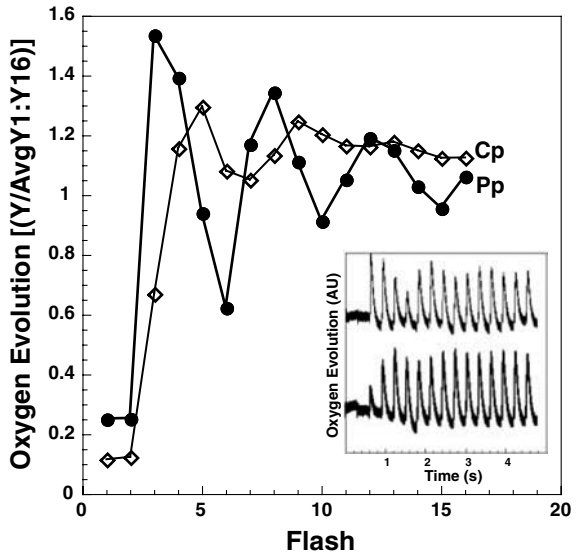

Figure 2. Flash-induced oxygen evolution. Oxygen flash yield (normalized by the average of all values) is plotted as a function of the flash number to show the oscillation pattern of PSII activity from *Ceratodon* (Cp) and *Physocmitrella* (Pp) protonemal tissue. The inset shows oxygen evolution data collected over the course of the experiment with saturating flashes of light given at 300 ms intervals. Cells were trained with 20 preflashes and then dark-adapted for 10 min prior to data collection. Top trace, *Physcomitrella*; bottom trace, *Ceratodon.*

Heterotrophic growth

Both *Physcomitrella* and *Ceratodon* were examined for their ability to grow in the absence of photosynthesis. The strains were cultured in the absence and presence of DCMU for 12 days. Mosses grown on normal moss growth medium (BCDA) are shown in Figure 3a. Comparison of the size and density of the plants showed that *Ceratodon* grew better than *Physcomitrella*. Figure 3b shows that growth in the presence of 0.5% glucose was comparable to that in its absence (Figure 3a). In Figure 3c, growth was drastically reduced in the presence of DCMU. *Physcomitrella* did not grow at all, but the original inoculum remained green. *Ceratodon* grew slowly in the presence of DCMU, even though there was an obvious increase in the amount of tissue, suggesting that *Ceratodon* was able to use glucose as an alternate carbon source when photosynthesis was blocked.

Physcomitrella and *Ceratodon* were also tested for their ability to grow in the absence of light. Figure 4a shows that in the dark, *Ceratodon* formed tall, white mounds of tissue whose filaments resembled etiolated seedlings. Dark-grown *Ceratodon* cells have been described previously with respect to negative gravitropism (for example, Schwuchow et al. 1990; Walker and Sack 1990). *Physcomitrella* did not grow in the dark and the tissue turned brown. Several other carbon sources (sucrose, fructose, and acetate) were tested, but glucose best supported the growth of *Ceratodon* in the absence of photosynthesis, even though there was some growth in the presence of sucrose (data not shown).

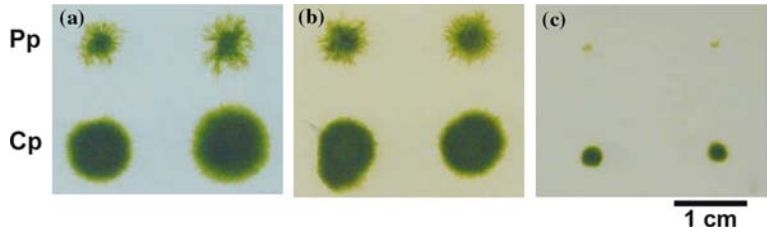

Figure 3. Growth of mosses in the presence of PS II inhibitors. (a) *Physcomitrella* (Pp) and *Ceratodon* (Cp) after 12 days of growth on BCDA medium. (b) Growth on media supplemented with 0.5% glucose. (c) Growth on media with glucose and 10 μM DCMU to block photosynthesis.

Greening

After determining that *Ceratodon* could grow in the dark, it was important to examine its ability to return to its green, and photosynthetic state in the presence of light. Figure 4b shows that a plant grown in the dark turned green after only 24 h of light. Furthermore, the filaments that were reaching up and away from the growth cone in the dark fell back towards the media after being exposed to the light. The cellular structure of dark-grown and greening plants was also examined using transmission electron microscopy (Figure 5). In Figure 5a, a typical light-grown cell is shown. The three chloroplasts shown in this cross section are about one half of the cell diameter and contain thylakoid membranes arranged in granal stacks (7–10 membranes per stack) and stroma lamellae. Starch granules are present but small and few.

Figure 5b shows that tissue grown in the dark for 10 days had considerably smaller cells with thick walls and plastids contained many large starch granules. Prolamellar bodies, typically seen in dark-grown eukaryotic photosynthetic organisms, were not observed in any of the sections examined. The sizes of the plastids were typically one third of the cell diameter. After 2.5 h of greening, plastids showed considerable enlargement, contained a few unstacked thylakoid membranes, and contained fewer and smaller starch granules (Figure 5c). Figure 5d is an example of cells that had been greening for 23 h, which contained chloroplasts more closely resembling light-grown cells. Stacks of grana were evident, and there were fewer starch granules. After 23–24 h in the light, photosynthetic activity in the dark grown culture reached a level similar to light grown cells (data not shown). These data suggest that dark grown *Ceratodon* cells are able to quickly convert undifferentiated plastids into chloroplasts as has been observed in vascular plants, such as pea seedlings (Frances et al. 1992) and the green algae, *C. reinhardtii* Y1 strain (Ohad et al. 1967).

Mutant phenotypes

In order to determine if mosses could be useful for photosynthesis mutant studies, it was necessary to determine whether it is possible to isolate mutants with impaired photosynthetic activity. *Ceratodon* protoplasts were mutagenized by a UV treatment producing 95% kill, and the sur-

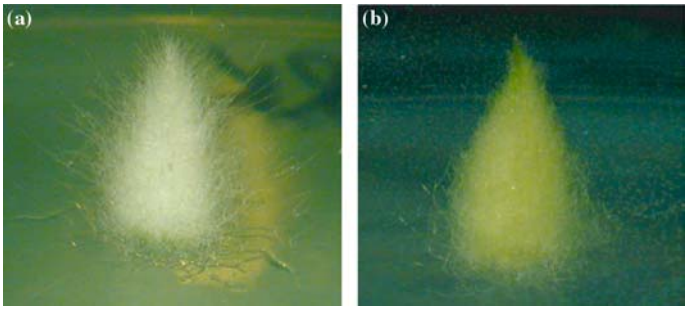

Figure 4. Greening of *Ceratodon* protonema. (a) A *Ceratodon* colony grown in the dark for 11 days. (b) After growth in the dark, the plate was moved into the light for 24 h.

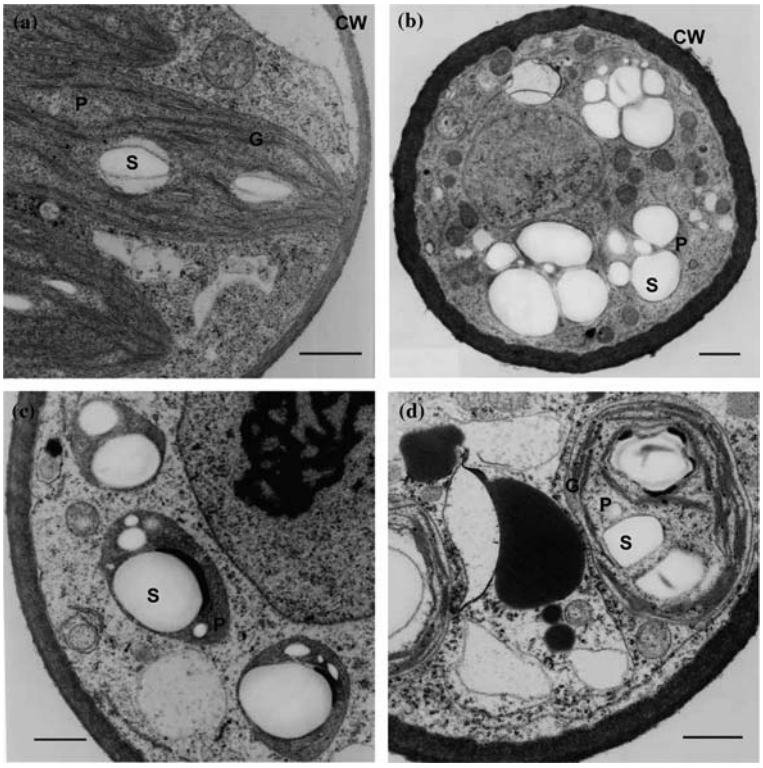

Figure 5. EM of greening *Ceratodon* protonema. (a) Electron micrograph of light-grown *Ceratodon* protonemal cell cross section. (b) Ten days growth in the dark. (c) Dark-grown cell after 2.5 h in the light. (d) Dark-grown cell after 23 h in the light. P, plastid; S, starch granule; G, grana stacks; CW, cell wall (bar = 1 μm).

viving plants were subjected to two different screens, one for photosynthetic activity and one for greening. Seven days following mutagenesis, 1456 *Ceratodon* plants were picked. Only 11 days later, 1052 (61%) grew to a size that allowed for fluorescence screening. The plants were screened using the FluorCam CCD camera described above. Twenty-five plants had apparent photosynthetic deficiencies, as summarized in Table 1. These plus eight other plants with potential photosynthesis deficiencies were subcultured onto media with and without glucose and tested for a second time. Fourteen (1% of total) of the plants retained their phenotypes through the second round of testing. One plant, 19-13, showed a substantially slower growth rate in the absence of glucose, indicating a severe impairment of the photosynthetic system. Figure 6 shows representative fluorescence traces from several classes of mutants isolated.

After the photosynthesis screen, the plants were subcultured onto fresh plates for the greening screen. The plates were kept in the dark for 10 days and then moved into the light. Plants were then observed for their ability to turn green within the normal 24-h period. Fifty greening mutants were observed, and of these 37 turned green slower than wild-type, and 23 greened faster than wild-type.

Discussion

Here we have reported several examples of *in vivo* biophysical analysis of photosynthetic activity in the mosses, *Physcomitrella* and *Ceratodon*, which illustrates their usefulness as tools of photosynthesis studies. As simple land plants, these organisms provide a model that grows faster, takes up less culture space, and is better suited for *in vivo* analysis than vascular plants. Even though mosses contain some of the attributes that make cyanobacteria as useful model organisms, such as *in vivo* analysis and targeted gene replacement (*Phys-*

Table 1. Photosynthetic mutants isolated from UV mutagenesis

Mutant[a]	F_0 value	F_v/F_m	F_s quenching
1.1-20	High	0.59	Normal
1.2-7	Normal	0.64	Slow
1.4-13[b]	Normal	0.72	Slow
1.4-9	Normal	0.64	Slow
1.13-3	Normal	0.52	Normal
1.13-6	High	0.49	Normal
1.14-19	Very High	0.55	Slow
1.14-29	High	0.56	Normal
1.16-25	High	0.46	Fast
1.16-33[b]	High	0.58	Slow
1.19-13[b]	Very High	0.31	ND[c]
1.21-9	High	0.50	Normal
1.23-4	Normal	0.58	Normal
1.24-6	Very High	0.60	Normal
1.11-13	Normal	low	Slow
1.11-31	Normal	low	Normal
2.1-13	Normal	0.77	Slow
2.2-3	Normal	0.57	Normal
2.2-22	Normal	0.57	Normal
2.2-2	Normal	0.60	Fast
2.3-3	Normal	0.56	Normal
2.5-8	High	0.57	Slow
2.7-17	Normal	0.69	Fast
2.8-14	Normal	0.51	Normal
2.9-25	Normal	Not available	Slow
Total: 25	10 high	18 low	4 fast, 9 slow

[a] Mutant numbers are derived from the round of picking plants, the plate and the plant location.
[b] The fluorescence traces for these samples are shown in Figure 6.
[c] ND, not detected because F_0 was too high.

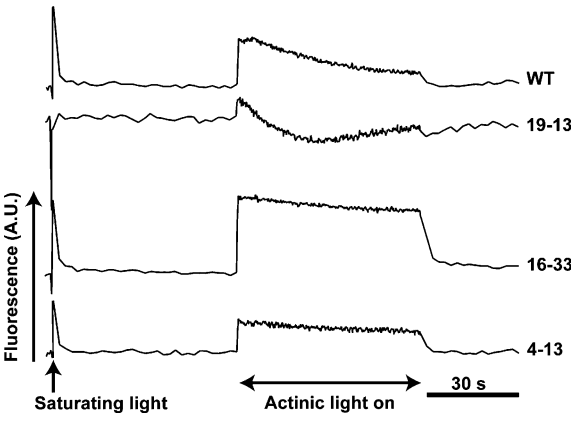

Figure 6. Fluorescence traces of UV-induced photosynthetic mutants. Representative traces of several mutants isolated after UV-induced mutagenesis of *Ceratodon* protoplasts. The measurement was performed the same as described in Figure 1c.

comitrella), the genomic organization and physiology is more similar to vascular plants.

Both *Ceratodon* and *Physcomitrella* exhibit photosynthetic fluorescence activity similar to that of vascular plants. Analytical fluorescence rise and decay kinetics can be measured *in vivo* in moss cells (Figures 1a, b); whereas such measurements in land plants are normally performed on isolated thylakoid membranes. The mosses are also amenable to CCD camera-based fluorescence measurements (Figure 1c), which makes screening large numbers of mutants easy. We were able to use fluorescence to screen UV-induced mutants and categorize photosynthesis-deficient mutations in *Ceratodon*. The observation that photosynthesis mutants and greening mutants can be maintained in this moss suggests that non-lethal disruptions in genes important to these processes can be supported in *Ceratodon*. Furthermore, flash-induced oxygen evolution measurements can also be performed on intact moss cells, as opposed to isolated chloroplasts or thylakoid membranes from other plant tissue. Figure 2 shows the flash pattern for oxygen evolution in moss cells. It is unclear why *Ceratodon* did not have the expected peak on the fourth flash. Further investigation as to the physiology of this organism may reveal details of S-state stability in PS II. These mosses provide the opportunity to study PS II donor side electron transport *in vivo*.

Greening provides an important tool for studying chloroplast formation and photosystem biogenesis. Because *Ceratodon* is able to grow in the dark, it is well suited for greening experiments. Here we identified two classes of greening mutants, those that are slower and those that are faster than wild-type cells. The slower class could be deficient in signaling components, or biogenesis components important for initiating chloroplast development in response to light. The faster class could be impaired in regulatory proteins important for maintaining undifferentiated plastids. Further study on *Ceratodon* greening will be useful for examining chloroplast biogenesis and the mechanism of thylakoid membrane formation.

There is a well-established community of researchers who have developed tools, such as an EST database and gene trap lines, for *Physcomitrella* studies, which makes this organism a useful tool for photosynthesis research. Furthermore, genome sequencing of *Physcomitrella* has begun,

which will further establish this organism as a useful model system. As described above, some photosynthesis experiments are more easily performed in *Ceratodon*, because it can be maintained in the absence of photosynthesis. Many techniques, such as DNA transformation and culture maintenance, are transferable between *Physcomitrella* and *Ceratodon*. Therefore, *Ceratodon* is well poised to be a useful tool for studying integral components of the photosynthesis apparatus. More interest in this organism will help push the genomic resources to the level of *Physcomitrella*. Until an alternate carbon source is found to support non-photosynthetic growth in *Physcomitrella*, this organism can be used for the study of auxiliary components of photosynthetic processes.

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