

Photoheterotrophic growth of *Physcomitrella patens*

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Abstract *Physcomitrella patens* is a model bryophyte representing an early land plant in the green plant lineage. This organism possesses many advantages as a model organism. Its genome has been sequenced, its predominant life cycle stage is the haploid gametophyte, it is readily transformable and it can integrate transformed DNA into its genome by homologous recombination. One limitation for the use of *P. patens* in photosynthesis research is its reported inability to grow photoheterotrophically, in the presence of sucrose and the Photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which prevents linear photosynthetic electron transport. In this communication we describe the facile isolation of a *P. patens* strain which can grow photoheterotrophically. Additionally, we have examined a number of photosynthetic parameters for this strain grown under photoautotrophic, mixotrophic (in the presence of sucrose) and photoheterotrophic conditions, as well as the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-inhibited state. The ability to grow *P. patens* photoheterotrophically should significantly facilitate its use in photosynthetic studies.

Introduction

The moss *Physcomitrella patens* is an important developing model organism. As a bryophyte, it represents a

non-vascular land plant, the earliest representatives of which were present at least 350–400 million years ago (Hueber 1961). The predominant life cycle stage of these organisms is the haploid gametophyte. The *P. patens* genome has been sequenced (Rensing et al. 2008), the organism is transformable and can integrate exogenous DNA into its genome via homologous recombination (Schaefer and Zrýd 1997). The ability to specifically target genes for knockout and replacement has proved a very useful characteristic of this organism. Recently, *P. patens* has been used in an increasing number of studies examining plant evolution, physiology and metabolism (Cove 2005; Cove et al. 2006).

One limitation on the use of *P. patens* in the field of photosynthesis is its reported inability to grow heterotrophically (Thornton et al. 2005), i.e. in the absence of linear chain photosynthetic electron transport when supplied with a carbon source. These authors observed no growth of *P. patens* when gametophytic explants were transferred to media containing 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 0.5 % glucose. They also tested a number of other carbon sources, none of which could apparently support heterotrophic growth. The two organisms which have proved, arguably, among the most useful for photosynthesis research, are the cyanobacterium *Synechocystis* sp. PCC 6803 (henceforth *Synechocystis* 6803) and *Chlamydomonas reinhardtii* (henceforth *Chlamydomonas*); both can utilize exogenous carbon sources to support growth in the absence of linear chain electron transport. *Synechocystis* can be cultured photoheterotrophically in the presence of the Photosystem II (PS II) herbicide DCMU when supplied with glucose (Williams 1988). This has allowed the isolation of numerous PS II mutants (Williams 1988; Vermaas et al. 1987; Bricker et al. 1998; Pakrasi et al. 1989). This organism can also

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grow very slowly via light-activated heterotrophic growth on glucose in darkness if supplied with a brief pulse of blue light on a daily basis (Anderson and McIntosh 1991). *Chlamydomonas* can grow heterotrophically in the dark when supplied with acetate (Rochaix 1987). Mutants completely lacking PS II have also been isolated and characterized in this organism (de Vitry et al. 1989). Consequently, the ability to grow heterotrophically allows the genetic manipulation of genes involved in photosynthesis and the recovery of otherwise lethal mutations in the photosynthetic apparatus.

Interestingly, a number of reports have indicated that *P. patens* can utilize sucrose to supplement photosynthetic growth. For instance, gametophytic tissue grown in the presence of sucrose grows more rapidly than in its absence (Frank et al. 2005). Additionally, if sucrose is provided under dark growth conditions, the growth of caulonemal tissue, which contains few chloroplasts, is enhanced, while the growth of chloronemal tissue, which contains abundant chloroplasts, is suppressed (Cove et al. 1978). Under low light intensities or very short day lengths the presence of sucrose enhances the growth rate of chloronemal tissue (Cove et al. 1978). Finally, putative sucrose uptake transporters are present in the *P. patens* genome. Two type IIA and three type III sucrose transporters appear to be present, although their subcellular localization and physiological roles have yet to be determined. It has been suggested that sucrose uptake transporters may be important for scavenging sucrose from the environment in non-vascular plants (Reinders et al. 2012). All of these studies seem to indicate that some capacity for the utilization of exogenously supplied sucrose is present in wild-type *P. patens*.

Materials and methods

Plant materials and growth conditions

Physcomitrella patens, strain Gradsen was maintained on agar plates containing BCD medium (Cove et al. 2009) + 1 % sucrose prior to the initiation of these experiments. For the examination of growth under different culture conditions, 1.5–2 mm gametophytic explants were transferred to agar plates containing BCD medium (photoautotrophic growth), BCD medium + 1 % sucrose (mixotrophic growth), BCD medium + 1 % sucrose + 10 μ M DCMU (photoheterotrophic growth) or BCD medium + 10 μ M DCMU (PS II-inhibited). The growth temperature was 22 °C with continuous illumination (60 μ mol photons $\text{m}^{-2} \text{s}^{-1}$). Except where indicated, plants were grown for 5–12 weeks prior to characterization.

Fluorescence and spectroscopic measurements

For all of the fluorescence and spectroscopic measurements, gametophytes were dark-incubated for 5 min before initiation of the experiments. OJIP fluorescence induction and non-photochemical quenching (NPQ) measurements were performed using a Photon Systems Instruments FluorCam 800MF. Both measuring and saturating flashes are provided by computer-controlled photodiode arrays. Data analysis was performed using proprietary Photon Systems Instruments software. The steady-state P_{700} and cytochrome *f* measurements were performed using a Joliot-Type Spectrophotometer (JTS-10, Bio-Logic Scientific Instruments) operating in the absorbance mode using the ‘pulse of dark’ method. For the P_{700} measurements, samples were illuminated with a broadband actinic orange light source with a peak of 630 nm for 5 s illumination and the absorbance changes at 705 nm monitored P_{700} oxidation. At the end of the 5 s actinic illumination period, P_{700}^{+} was reduced in the dark. The broadband actinic light source excites both PS II and PS I. For the cytochrome *f* measurements the same actinic illumination protocol was used and absorbance data were collected at 546, 554, 563 and 573 nm. Data were analyzed using proprietary software provided by Bio-Logic Scientific Instruments and Origin version 6.1 (OriginLab, Corp.).

Electrophoresis and protein detection

Thylakoids from gametophytes were isolated by grinding in a glass homogenizer using a buffer containing 20 mM Tricine–NaOH, pH 8.4, 0.45 M sorbitol, 10 mM EDTA, 0.1 % BSA and 1 % polyvinylpyrrolidone. The homogenate was filtered through two layers of Miracloth (Calbiochemical Co.) and the thylakoids pelleted at $2,500\times g$ for 5 min. The thylakoid pellet was resuspended in a small volume of 0.3 M sorbitol, 20 mM Tricine–NaOH, pH 7.6 and 5 mM MgCl_2 and frozen at -80°C until use. The chl concentration was determined by the method of Arnon (1949). Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LiDS-PAGE) was performed under conditions described by Delepelaire and Chua (Delepelaire and Chua 1979) using gradient 12.5–20 % polyacrylamide gels. The resolved polypeptides were electroblotted onto PVDF membranes (Immobilon-P, Millipore Corp.). After blocking for 2 h with 5 % nonfat dry milk in TS buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.4), the blots were washed extensively with TS buffer and then incubated with diluted primary antibody in TS buffer + 1 % bovine serum albumin overnight. This was followed by washing in TS buffer and incubation with either anti-rabbit or anti-mouse IgG-peroxidase conjugate (Sigma) diluted in TS buffer + 1 % bovine serum albumin. After washing in TS buffer, the labeled protein was detected using chemiluminescence

(Super Signal West Pico, Pierce Chemical Co.) and semi-quantified as described previously (Yi et al. 2009).

Results and discussion

Initially, we were able to fully replicate the results of Thornton et al. (2005). In the presence of 10 μ M DCMU, no growth of gametophytic tissue was observed on plates supplemented with glucose or a variety of other carbon sources (fructose, sucrose, acetate or pyruvate, all at three different concentrations, data not shown). These cultures were all started from parent cultures maintained autotrophically on standard BCD media in the absence of any additional carbon source.

We hypothesized that the use of sucrose by gametophytic tissue might require acclimatization to the exogenously supplied sugar. Consequently, *P. patens* cultures were maintained for 1 month on BCD medium + 1 % sucrose. Explants of gametophytic tissue (\approx 2 mm) grown in this manner were then plated onto various media. The results, after 8 weeks of incubation, are shown in Fig. 1. Luxuriant growth was observed on BCD medium, alone (photoautotrophic growth, Fig. 1a) and on BCD medium supplemented with 1 % sucrose (mixotrophic growth,

Fig. 1b). Interestingly, significant gametophytic growth was also observed for BCD medium containing 1 % sucrose + 10 μ M DCMU (photoheterotrophic growth, Fig. 1c). No growth was observed on BCD medium containing 10 μ M DCMU in the absence of sucrose (DCMU-inhibited state, Fig. 1d). It should be noted that microscopic observation of the explant borders of the photoheterotrophically grown tissue indicated that new chloronemal filaments were being formed as early as 1 week after transplantation (data not shown). Clearly the extent of the apparent photoheterotrophic growth of *P. patens* (Fig. 1c) is lower than observed for either photoautotrophically (Fig. 1a) or mixotrophically (Fig. 1b) grown cultures. This was not unexpected. Both, the photoheterotrophic growth of *Synechocystis* 6803 on glucose and the heterotrophic growth of *Chlamydomonas* on acetate also are much slower than observed for photoautotrophically or mixotrophically grown cultures.

Figure 2 shows the general morphology of the gametophytic tissue under these various growth conditions. The gametophylls of photoautotrophically (Fig. 2a), mixotrophically (Fig. 2b) and photoheterotrophically (Fig. 2c) grown tissue all appear very similar. These are typically bright green and have the same general shape. However, those from mixotrophically grown tissue are somewhat larger

Fig. 1 Illustrated is the growth of 1.5–2 mm gametophytic explants after 8 weeks; all panels are at the same magnification. **a** Photoautotrophic growth, **b** mixotrophic growth, **c** photoheterotrophic growth and **d** PS II-inhibited gametophytes. At 8 weeks, the DCMU-inhibited gametophytes have fully bleached and are apparently dead. Clearly, photoheterotrophic growth is slower than photoautotrophic or mixotrophic growth conditions. This was fully expected and is similar to that observed in *Synechocystis* 6803 and *Chlamydomonas* grown under analogous growth conditions

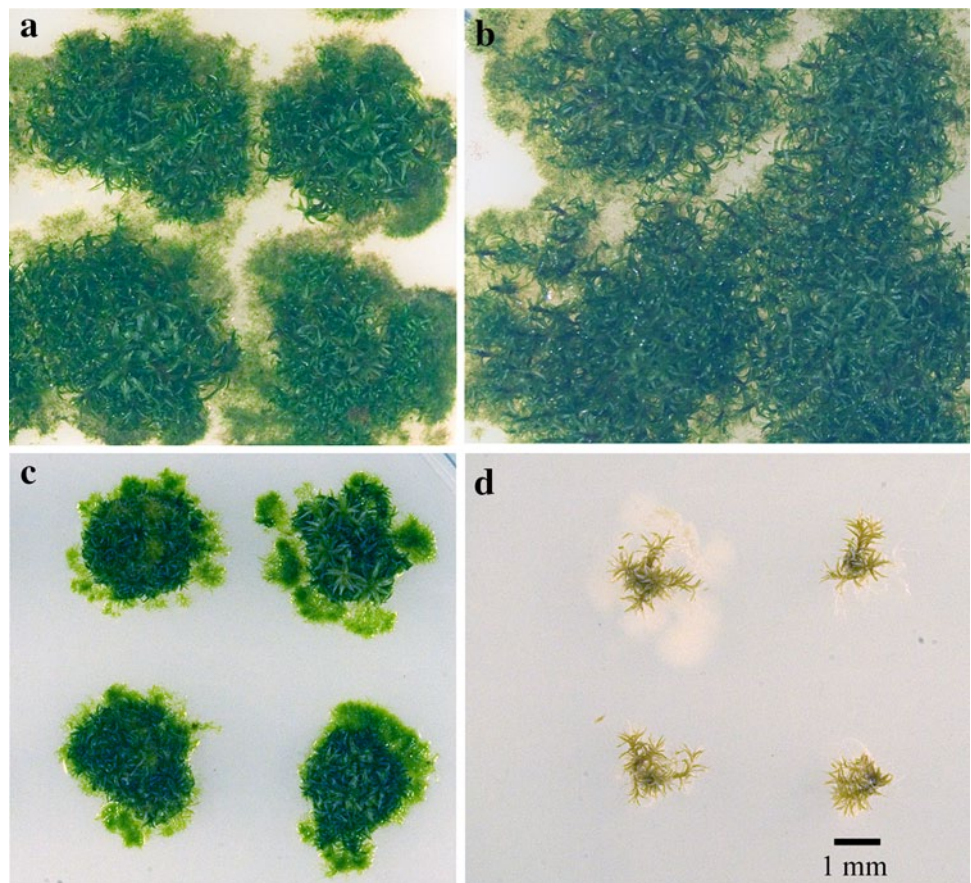
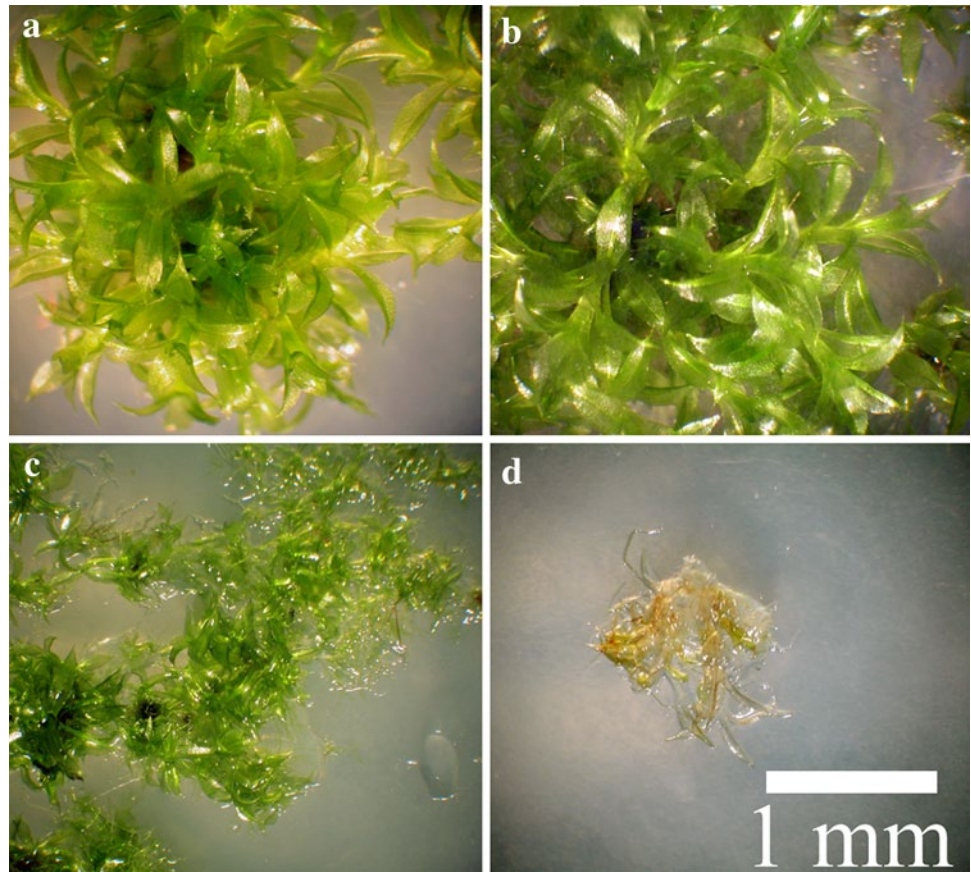


Fig. 2 Details of the appearance of gametophytes cultured under the various growth conditions; all *panels* are at the same magnification. **a** Photoautotrophic growth, **b** mixotrophic growth, **c** photoheterotrophic growth and **d** PS II-inhibited gametophytes. The gametophylls of the photoheterotrophically grown gametophytes are smaller than those grown under photoautotrophic or mixotrophic conditions



than those grown autotrophically; the gametophylls from photoheterotrophically grown tissue are quite small. The DCMU-inhibited tissue (Fig. 2d) is bleached and, as noted above, no growth was observed.

These results indicated that *P. patens* was capable of growth on media containing sucrose in the presence of DCMU. This apparent photoheterotrophic growth could, however, be due to a spontaneous mutation which yielded DCMU-resistant mutant gametophytes. Such mutants have been isolated in the D1 protein of PS II, which bears the Q_B binding site and are known in both *Synechocystis* 6803 (Bouyoub et al. 1993; Dalla-Chiesa et al. 1997) and *Chlamydomonas* (Galloway and Mets 1982; Erickson et al. 1984). It is also formally possible

that in the presence of sucrose, DCMU uptake is inhibited. In either of these instances it would be expected that the tissue exhibiting apparent photoheterotrophic growth would possess relatively normal PS II electron transport characteristics, with electrons being transported to Q_B , the plastoquinone pool and beyond even in the presence of DCMU. Table 1 shows the analysis of an OJIP fluorescence induction experiment (Strasser et al. 2000) performed on *P. patens* grown for 12 weeks under the different test conditions. Please note that after 12 weeks, gametophytes transferred to the DCMU-inhibited medium were bleached and dead (Fig. 1d). For the fluorescence induction experiment, gametophytes were characterized after 2 weeks of incubation on this medium. At this time

Table 1 Fluorescence parameters of *Physcomitrella* gametophytes grown under various conditions

Growth condition	F_v/F_M	ABS/RC	TR _Q /RC	ET _Q /RC	DI _Q /RC	Ψ_0	Φ_{E_0}
Autotrophic	0.65 ± 0.06	4.22 ± 0.07	2.58 ± 0.05	1.03 ± 0.04	1.64 ± 0.04	0.39 ± 0.02	0.24 ± 0.01
Mixotrophic	0.66 ± 0.07	3.27 ± 0.13	2.25 ± 0.10	0.86 ± 0.04	1.01 ± 0.10	0.38 ± 0.02	0.27 ± 0.02
Photoheterotrophic	0.19 ± 0.09	42.34 ± 8.69	3.19 ± 0.39	−0.12 ± 0.38	39.18 ± 8.64	−0.22 ± 0.19	0.02 ± 0.01
DCMU-inhibited ^a	0.23 ± 0.03	24.2 ± 17.6	4.78 ± 1.67	2.04 ± 1.89	19.40 ± 16.69	0.38 ± 0.81	0.01 ± 0.02

Fluorescence induction curves were analyzed by the methods described in Strasser et al. (2000), $n = 9$, mean ± 1.0 SD

^a These gametophytes were assayed after 2 week. After 12 weeks of treatment gametophytes treated with this condition were dead

point, these gametophytes had not bleached and were still quite green.

Only modest differences are evident between autotrophically and mixotrophically grown gametophytes. While the quantum yield for energy trapping by PS II (F_V/F_M) is slightly higher in the mixotrophically grown gametophytes, the absorption of photons on a reaction center basis (ABS/RC), their trapping by PS II (TR/RC), electron transport beyond Q_A^- (ET/RC) and energy dissipation (DI/RC) are all somewhat higher in the gametophytes grown autotrophically. No differences were observed for the quantum yield of electron transport (ϕ_{E0}) or the efficiency of a trapped photon leading to productive electron transport past Q_A^- (Ψ_0). Overall, the differences between the fluorescence parameters observed under these two different growth conditions are quite small. The gametophytes grown under photoheterotrophic conditions and the DCMU-inhibited gametophytes, however, exhibited highly altered fluorescence kinetics. The ET_0/RC and Ψ_0 is ≈ 0 under these conditions, indicating that in the presence of DCMU, no electron transport can occur past Q_A^- . The ABS/RC and DI/RC were very high. The small apparent number of fully functional reaction centers, indicated by a very low F_V/F_M , leads to these high values. Clearly, the gametophytes grown under photoheterotrophic conditions and the DCMU-inhibited gametophytes exhibit very similar fluorescence characteristics. These fluorescence parameters indicate that the photoheterotrophically grown and the DCMU-inhibited gametophytes have no capacity for electron transport past Q_A^- , and electron transport appears fully inhibited by DCMU. Consequently, true photoheterotrophic growth has been observed in *Physcomitrella*.

The observation of a low F_V/F_M in gametophytes grown in the presence of DCMU is quite interesting. This could be due to a loss of variable fluorescence brought about by either an increase in F_0 , a decrease in F_M , or a combination of these two conditions. Figure 3 illustrates the unprocessed fluorescence data obtained from the different growth states. Both the autotrophically and mixotrophically grown gametophytes exhibited relatively high F_M and relatively low F_0 values. The photoheterotrophically grown and DCMU-inhibited gametophytes exhibited a similarly high F_M as observed under the other growth conditions; however, these also exhibited a high F_0 . These results indicate that while a similar number of reaction centers were present under all growth conditions, the number of reaction centers which could carry out successful charge separation was much smaller in the gametophytes grown photoheterotrophically and those which were DCMU-inhibited.

We also examined the function of PS I and the cytochrome b_6/f complex under the different growth conditions. Figure 4 illustrates the steady-state oxidation (Fig. 4a) and reduction (Fig. 4b) kinetics of P_{700} . The

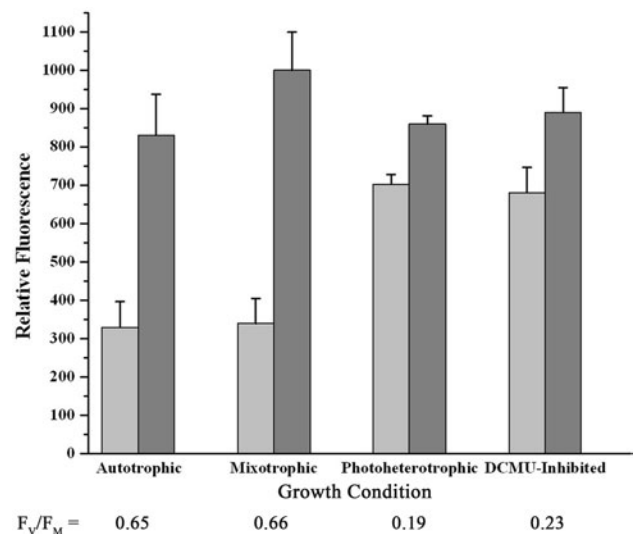


Fig. 3 Raw fluorescence data for the gametophytes grown under the different growth conditions. Autotrophically and mixotrophically grown gametophytes exhibited a low initial level of fluorescence (F_0) and a high level of maximal fluorescence (F_M). While the photoheterotrophically grown and DCMU-inhibited gametophytes also exhibited a relatively high F_M , these had a high F_0 . This leads to a low F_V/F_M value in these gametophytes. Plotted are means ± 1 SD, $n = 9$; light gray, F_0 ; dark gray, F_M . For reference, the F_V/F_M values are shown below

steady-state oxidation kinetics are very similar for the gametophytes grown under all conditions. These results indicate that PS I charge separation was not perturbed under mixotrophic or photoheterotrophic growth conditions. Autotrophically and mixotrophically grown gametophytes exhibited similar rates of P_{700}^+ reduction, while this was markedly slowed under photoheterotrophic growth conditions. This was expected since in the presence of DCMU both the plastoquinone and the plastocyanin pools are in a predominately oxidized state; consequently only a small amount of reduced plastocyanin is available to donate electrons to PS I. Similar results were observed for the cytochrome b_6/f complex as monitored by steady-state cytochrome f oxidation (Fig. 4c) and reduction (Fig. 4d). The rate of cytochrome f oxidation was similar under all growth conditions while the reduction of oxidized cytochrome f was seriously retarded during photoheterotrophic growth, again due to the oxidized plastoquinone pool present under photoheterotrophic growth conditions. The rates of oxidation and reduction for both P_{700} and cytochrome f are summarized in Table 2.

It is possible that the presence of sucrose during mixotrophic growth could lead to an alteration in the rate or extent of NPQ development. This could theoretically occur if there were either a direct or indirect redox coupling between the respiratory and photosynthetic electron

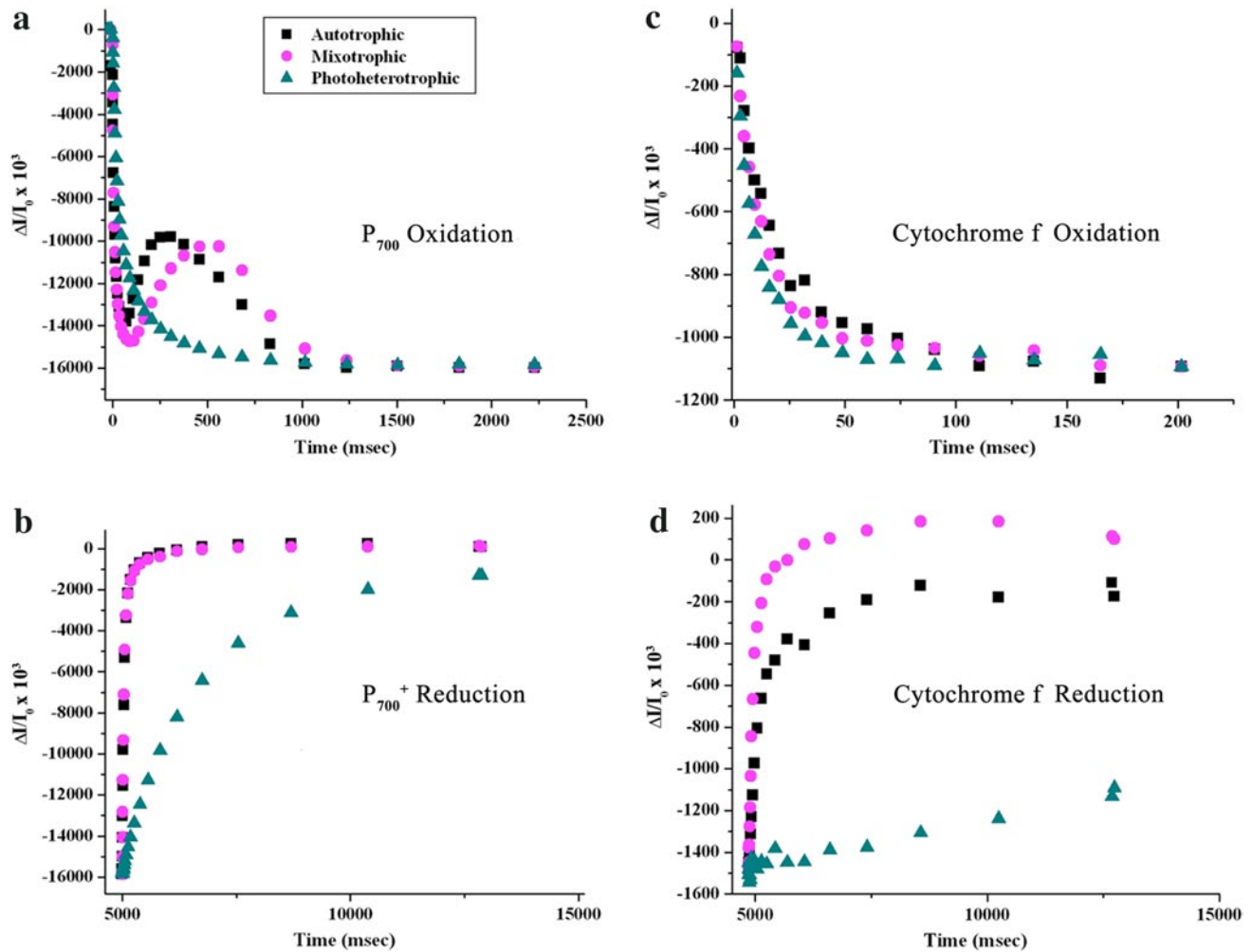


Fig. 4 Steady-state P_{700} and cytochrome f oxidation and reduction kinetics. For the P_{700} measurements (**a**, **b**), orange actinic light ($560 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was provided and the relative absorption was measured at 705 nm. For the cytochrome f measurements (**c**, **d**), orange actinic light ($560 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) was provided and

relative absorption was measured at 546, 554, 563 and 573 nm. These data were then deconvoluted to yield the cytochrome f absorption. Data were normalized to the value at time 0 representing the onset of actinic illumination (**a**, **c**) and at 5 s representing the cessation of actinic illumination (**b**, **d**)

Table 2 Steady-state oxidation and reduction characteristics of cytochrome f and P_{700} , $n = 4$, mean ± 1.0 SD

Growth condition	Cytochrome f		P_{700}	
	$t_{1/2}$ oxidation (ms)	$t_{1/2}$ reduction (ms)	$t_{1/2}$ oxidation (ms)	$t_{1/2}$ reduction (ms)
Autotrophic	41 ± 4	151 ± 27	30 ± 10	34 ± 3
Mixotrophic	41 ± 3	84 ± 10	24 ± 10	30 ± 4
Photoheterotrophic	34 ± 5	$2,700 \pm 1,300$	51 ± 16	$1,100 \pm 100$

transport chains. Figure 5 illustrates the result of an NPQ experiment performed at a moderate ($365 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) actinic light intensity. Both the development of NPQ upon onset of the actinic illumination and its dissipation upon cessation of illumination were nearly identical in both the autotrophically and mixotrophically grown gametophytes. It should be noted that no NPQ development was observed in the photoheterotrophically grown

gametophytes (data not shown). These results indicate that no effect on NPQ was evident under mixotrophic growth conditions.

Finally, a number of thylakoid proteins associated with the different photosynthetic electron transport complexes were also examined under the various growth conditions (Fig. 6). In most instances, the examined proteins accumulated to somewhat lower levels (50–80 %) under

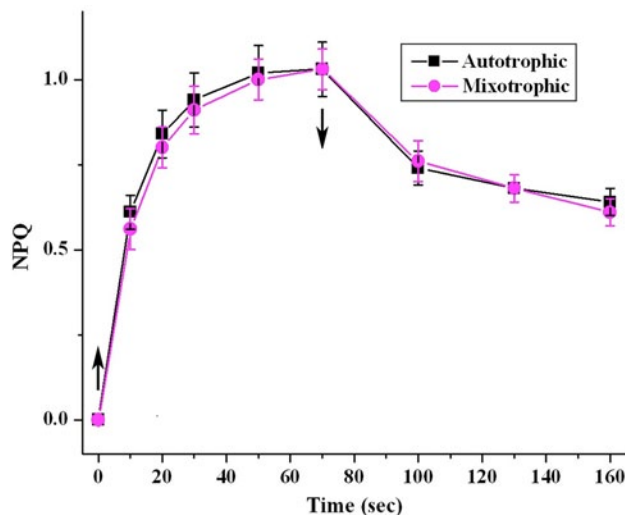


Fig. 5 NPQ development and relaxation of autotrophically and mixotrophically grown gametophytes. Measurements were taken at an actinic light intensity of $365 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Arrows indicate actinic light on and off. Photoheterotrophically grown and DCMU-inhibited gametophytes exhibited no NPQ. Plotted are means ± 1 SD; $n = 9$

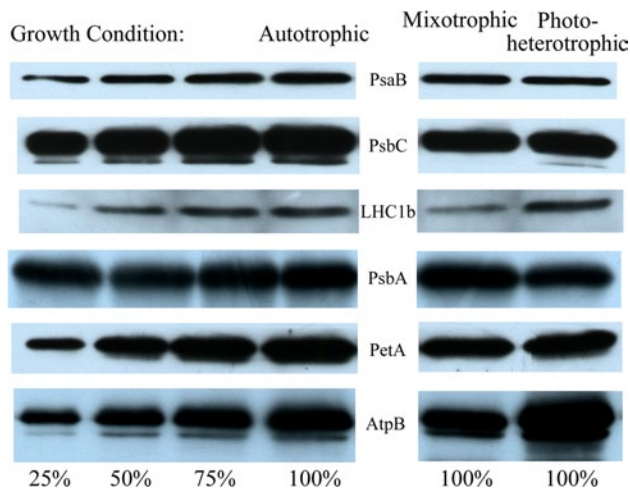


Fig. 6 Thylakoid proteins from autotrophically, mixotrophically and photoheterotrophically grown gametophytes were separated by LiDS-PAGE and identified by immunoblotting with specific antibodies. *Left panel* dilutions of the proteins from autotrophically grown gametophytes; *right panel* proteins from mixotrophically and photoheterotrophically grown gametophytes. The 100 % lanes contain $3 \mu\text{g}$ chlorophyll

mixotrophic and photoheterotrophic conditions when compared with autotrophically grown gametophytes. One exception was the AtpB protein, which accumulated to high levels during photoheterotrophic growth. This observation may indicate that the chloroplast ATP synthase accumulates under this growth condition. This, however, must be tested. While it is clear that genetic disruption of individual

subunits of the ATP synthase leads to a coordinated down-regulation of the other subunits (Dal Bosco et al. 2004; Drapier et al. 1992; Gatenby et al. 1989), the converse may not be generally true. Indeed, overexpression of the γ -subunit does not lead to increased abundances of other ATP synthase subunits (Dal Bosco et al. 2004).

Conclusions

We have demonstrated the ability to grow *P. patens* gametophytes photoheterotrophically in the presence of sucrose as a carbon source and the PS II inhibitor DCMU. Our studies indicate that successful photoheterotrophic growth requires prior acclimatization of *P. patens* to growth on sucrose-containing medium. Earlier, it had been shown that the moss *Ceratodon purpureus* can grow photoheterotrophically using glucose as a carbon source (Thornton et al. 2005). The use of *C. purpureus* to examine photosynthetic processes, however, is problematic. The draft genomic sequence is currently in the analysis stage and has not yet been released. Additionally, the molecular tools for manipulating genes in *C. purpureus* are not as well developed as those for *P. patens*. More critically, the function and/or stability of PS II appears to be different in *C. purpureus* than in *P. patens* and all other oxygenic photosynthetic organisms which have been studied. All wild-type organisms previously examined in flash oxygen yield experiments including higher plants (Joliot et al. 1969; Kok et al. 1970), cyanobacteria (Burnap et al. 1992), green algae (Jursinic 1979) and the moss *P. patens* (Thornton et al. 2005) exhibit a maximum oxygen yield on the third saturating flash after an extended dark incubation. This indicates that the oxygen-evolving complex is predominantly in the S_1 oxidation state (Kok et al. 1970). *C. purpureus*, however, exhibits a maximum yield on the fifth flash (Thornton et al. 2005). Taken at face value this indicates that the oxygen-evolving complex is in the S_1 state, usually associated with damaged oxygen-evolving complexes. Also, examination of the flash oxygen yield pattern for *C. purpureus* (Thornton et al. 2005) indicates that its oxygen yield oscillations are much more highly dampened than observed for other oxygenic organisms. This result indicates misses, double hits or deactivations (or a combination of these) are more prevalent in *C. purpureus* than in other oxygenic organisms. The basis for these differences is unknown although one can speculate that PS II from *C. purpureus* is more labile during thylakoid membrane isolation.

Our results indicate that during photoautotrophic and mixotrophic growth the photosynthetic characteristics of *P. patens* are very similar. The fluorescence characteristics (primarily probing PS II), as well as oxidation and reduction rates of both cytochrome *f* and P_{700} , and the rate of

development and dissipation of NPQ all indicate that only small differences exist between these two growth states. The observed differences in the protein composition of the thylakoid membrane are also quite modest. The differences observed for gametophytes grown under photoheterotrophic conditions are all consistent with loss of PS II function in the presence of DCMU. This is highlighted by the similarities between photoheterotrophically grown gametophytes and those which are DCMU-inhibited. While, in general, the alteration of the protein complement in thylakoids from photoheterotrophically grown gametophytes is very similar to those grown mixotrophically, the possible upregulation of the ATP synthase will require further study. Finally, the demonstrated ability to grow *P. patens* photoheterotrophically should augment and extend the utility of this model organism for use in photosynthesis research.

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