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Evaluation of visible-light wavelengths that reduce or oxidize the plastoquinone pool in green algae with the activated F_0 rise method

H. MATTILA[*], V. HAVURINNE[*,**], T. ANTAL[*,***,+], and E. TYYSTJÄRVI[*,+]

Molecular Plant Biology, Department of Life Technologies, University of Turku, 20014 Turku, Finland[*]
Present address: *ECOMARE, CESAM, Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal*[**]
Permanent address: *Laboratory of Integrated Ecological Research, Pskov State University, 180000 Pskov, Russia*[***]

Abstract

We recently developed a chlorophyll *a* fluorescence method (activated F_0 rise) for estimating if a light wavelength preferably excites PSI or PSII in plants. Here, the method was tested in green microalgae: *Scenedesmus quadricauda*, *Scenedesmus ecornis*, *Scenedesmus fuscus*, *Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, and *Ettlia oleoabundans*. The *Scenedesmus* species displayed a plant-like action spectra of F_0 rise, suggesting that PSII/PSI absorption ratio is conserved from higher plants to green algae. F_0 rise was weak in a strain of *C. reinhardtii*, *C. sorokiniana*, and *E. oleoabundans*. Interestingly, another *C. reinhardtii* strain exhibited a strong F_0 rise. The result indicates that the same illumination can lead to different redox states of the plastoquinone pool in different algae. Flavodiiron activity enhanced the F_0 rise, presumably by oxidizing the plastoquinone pool during pre-illumination. The activity of plastid terminal oxidase, in turn, diminished the F_0 rise, but to a small degree.

Keywords: *Chlamydomonas*; chlorophyll fluorescence; far-red acclimation; FlvB protein; *Scenedesmus*.

Introduction

Nowadays, green algae are of keen interest to the scientific community due to the great potential for photosynthetic production of biofuels, hydrogen gas, carotenoids, omega-3 fatty acids, and other useful compounds (for reviews, *see* Stensjö *et al.* 2018, Bolatkhan *et al.* 2019, Petrova *et al.* 2020, Bhatia *et al.* 2021). Optimization of biotechnological production systems requires a deep understanding of the effects of light on rearrangements of metabolism and regulatory networks, and in particular, on the regulation of photosynthetic processes and energy balance in the algal cell. Knowledge of spectral preferences of PSI and PSII in algae can provide valuable information on the light-dependent regulation of electron fluxes through PSII and PSI, and can therefore be employed in algal biotechnology

Highlights

- F_0 rise fluorescence can be used to probe PSII/PSI absorption ratio in green algae
- *Scenedesmus quadricauda* shows a similar F_0 rise spectra as higher plants
- Flavodiiron proteins contribute to the oxidation of the plastoquinone pool in white light

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[+]Corresponding authors
e-mail: taras_an@mail.ru (T. Antal)
esatyy@utu.fi (E. Tyystjärvi)

Abbreviations: Chl – chlorophyll; F_0 rise – post-illumination rise of chlorophyll *a* fluorescence; FR – far-red; flv – flavodiiron; FWHM – full width at half maximum; LL – low light; ML – measuring light; PAM – pulse amplitude modulation; PG – propyl gallate; PQ – plastoquinone; PTOX – plastid terminal oxidase; SP – saturating pulse.

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to optimize product yield, as previously shown, *e.g.*, for hydrogen photoproduction in *Chlamydomonas reinhardtii* (Antal *et al*. 2016).

Recently, we developed a fluorescence protocol for higher plants to screen the capacity of different wavelengths of light to excite preferentially PSI or PSII and, consequently, to oxidize or reduce the thylakoid membrane plastoquinone (PQ) pool (Mattila *et al*. 2020). The method exploits the transient post-illumination rise of chlorophyll (Chl) *a* fluorescence (the F_0 rise) that reflects reduction and subsequent oxidation of the plastoquinone (PQ) pool in the dark by stromal reductants *via* the machinery of cyclic electron flow (Mills *et al*. 1979, Endo *et al*. 1997, Field *et al.* 1998, Shikanai *et al*. 1998). In the activated F_0 rise method, a light-acclimated leaf is first illuminated with moderate actinic light for a few minutes. Then, this pre-illumination is changed to a weak monochromatic light (called activating light), and changes in Chl *a* fluorescence yield are probed with short pulses of the measuring light of a pulse amplitude modulation (PAM) fluorometer (Mattila *et al*. 2020). Activating light favoring PSII excitation over PSI excitation induces a high F_0 rise because oxidation of Q_A^- is slowed down by the accumulation of PQH_2 and because light favoring PSII enhances the formation of Q_A^-. On the contrary, activating light exciting preferentially PSI favors the oxidation of the PQ pool while little Q_A^- is formed, and hence the F_0 bump is suppressed or not observed at all. In *Arabidopsis thaliana*, the activated F_0 rise method is a sensitive indicator of the excitation balance between PSII and PSI; wavelengths favoring PSII reduce the plastoquinone (PQ) pool, at moderate intensity, and wavelengths favoring PSI oxidize the PQ pool (Mattila *et al*. 2020). The same wavelengths that cause oxidation or reduction of the PQ pool in plants also function in *C. reinhardtii*, but the variation in the redox state of the PQ pool is not as large in the alga as in the plant (Virtanen and Tyystjärvi 2022). In photosynthetic organisms harboring functional flavodiiron (Flv) proteins in the chloroplasts (Ilík *et al.* 2017), Flv activity is expected to affect the F_0 rise by depleting the stromal reductants.

In plants and green algae, the redox state of the PQ pool controls state transitions, which modify the energy balance between PSI and PSII (Allen *et al*. 1981, Vener *et al.* 1997, Finazzi *et al*. 2001, Depège *et al*. 2003, Nawrocki *et al*. 2016) and regulates nuclear and chloroplast gene expression (Escoubas *et al*. 1995, Pfannschmidt *et al.* 1999, Schönfeld *et al*. 2004). Antenna structures are known to react to the balance of the photosystems (for a review, *see* Lazar *et al.* 2022). In natural conditions with polychromatic white light, extreme oxidation or reduction of the PQ pool is unlikely, as electron transfer reactions of both photosystems function. Therefore, methods to probe the redox state of the PQ pool without setting a strong bias toward either extreme (with the help of sudden high light, far-red light, or inhibitors of electron transfer) are needed to understand the PQ-based regulation *in vivo*.

The knowledge obtained from higher plants cannot be directly used with algae, because the outer light-harvesting complexes of PSI and PSII of the green alga *C. reinhardtii*, for example, have been shown to greatly differ from those of plants (Tokutsu *et al*. 2012, Kawakami *et al*. 2019, Kubota-Kawai *et al*. 2019). Further differences may exist in other algae, and therefore methods are needed for the determination of wavelengths preferentially exciting one photosystem over the other in algae. In addition, the reactions involved in transient alterations of the PQ redox state during the post-illumination period may differ between green algae and plants due to differences in cyclic and pseudo-cyclic electron flow pathways and because of more substantial chlororespiration in green algae than in plants (Antal *et al*. 2013; for reviews, *see* Alric 2010, Alric and Johnson 2017). These factors can influence the F_0 rise pattern, raising the question about possible limitations and applicability of the activated F_0 rise method in microalgae.

In the current work, we tested the F_0 rise protocol in six algal species. Microalgae from the genera *Scenedesmus*, *Chlamydomonas*, *Chlorella*, and *Ettlia* were chosen for the current study, as these algae are widely used as model organisms and in studies on the production of biofuels and molecular hydrogen. We found that *Scenedesmus* strains show explicit F_0 rise with a plant-like action spectrum, whereas *C. reinhardtii*, *Chlorella sorokiniana*, and *Ettlia oleoabundans* exhibited a reduced F_0 rise. In addition, the results indicate that flavodiiron activity greatly affects the redox state of the photosynthetic electron transfer chain during illumination, while the plastid terminal oxidase (PTOX) may play a minor role during the post-illumination period.

Materials and methods

Strains and growth conditions: *C. sorokiniana* (Shihira & R.W. Krauss) and three strains of *Scenedesmus* [*S. quadricauda* (Turpin) Brébisson, *S. ecornis* (Ehrenberg) Chodat, and *S. fuscus* (Kirchner) E. Hegewald] were ordered from *CCALA* (http://ccala.butbn.cas.cz). Three *C. reinhardtii* strains, 125 (137C), the FlvB deletion strain 242 208, and its wild type cc4533, were obtained from the Chlamydomonas center (http://www.chlamycollection.org). The *flvB* mutant is further described by Jokel *et al.* (2018). The oleaginous species *E. oleoabundans* (earlier known as *Neochloris oleoabundans*) was ordered from *UTEX* (1185) (http://utex.org).

All algal cultures were grown autotrophically in BG11 medium (Rippka *et al.* 1979) buffered to pH 7.5 with 20 mM Hepes–KOH, except for *C. reinhardtii*, which was cultivated autotrophically in high salt medium (HSM) (Sueoka 1960). All cultures were started with an OD_{730} = 0.1. Three experimental conditions, marked as A, B, and C, were used for the cultivation of the algae:

• Condition A. Semi-continuous cultivation in 1-L flasks with constant bubbling by air and stirring at 26°C under illumination from a fluorescent lamp at photosynthetic photon flux density (PPFD) of 140 μmol m^{-2} s^{-1}. The spectral distribution of the light is shown in Fig. 1S (*supplement*). The cell concentration of the culture was kept constant by daily measurement of OD_{730} and dilution

with fresh medium to 0.35. Due to the daily growth, OD_{730} fluctuated between 0.35 and 0.40. The measurements were done after 7 d of cultivation.

• Condition B. Batch cultivation in 100-ml Erlenmeyer flasks on a shaker (100 rpm) at 26°C under a white LED matrix (Fig. 1S) at PPFD of 200 µmol m^{-2} s^{-1} in an *Algaetron* (*PSI*, Czech Republic). The measurements were done after 7 d of cultivation after which OD_{730} had reached approximately 0.6.

• Condition C. Batch cultivation in 100-ml Erlenmeyer flasks on a shaker (100 rpm) at 26°C under a fluorescent lamp (Fig. 1S) at PPFD of 35 µmol m^{-2} s^{-1}. The measurements were done after 14 d of cultivation after which OD_{730} had reached approximately 0.8.

PPFD was measured with a wavelength-calibrated quantum sensor (*LiCor*, Lincoln, NE) and light spectra were measured with an STS-VIS spectrometer (*Ocean Insight*, USA).

F_0 rise measurements – activated F_0 rise: Chl *a* fluorescence was measured with a *PAM-101* fluorometer (*Heinz Walz GmbH*, Germany) according to the activated F_0 rise protocol described by Mattila *et al.* (2020). An algal culture was pre-incubated for 1 h in weak white light (PPFD of 5–10 µmol m^{-2} s^{-1}) at room temperature. Algae were then collected on a glass fiber filter (with a diameter of 2.4 cm; *VWR*) with a final Chl concentration of 70–75 mg m^{-2}; Chl was extracted by at least 24-h incubation in methanol at 4°C in the dark (Inskeep and Bloom 1985) from separate cultures with known OD to estimate the needed algal volume. Then, a weak red measuring light (ML) was switched on using the frequency of 100 kHz. After 20 s, a saturating pulse (SP) (PPFD of 5,000 µmol m^{-2} s^{-1}, 0.8 s) was fired and an actinic light (PPFD of 50 µmol m^{-2} s^{-1}) was switched on simultaneously, as shown in Fig. 2S (*supplement*). A *Walz KL-1500* illuminator, equipped with a halogen bulb was used as a light source for this pre-illumination (for the spectra, *see* Fig. 1S) unless otherwise specified. After 180 s of the pre-illumination, a second SP was fired to estimate the magnitude of nonphotochemical quenching (NPQ), which can interfere with the F_0 rise measurements. After a further 60 s of illumination, the pre-illumination and ML were switched off and a monochromatic activating light (PPFD of 2.5 µmol m^{-2} s^{-1}), defined with a 10-nm full width at half maximum (FWHM) line filter (*Corion*, *Newport Corp*., Irvine, CA), was switched on. From here on, the ML was used in a chopped mode using cycles of 0.6 s on/5 s off unless otherwise mentioned. Activating light of 660 or 700 nm (FWHM 10 nm; *Corion*) that functions as PSII or PSI light, respectively, in *A. thaliana* (Mattila *et al*. 2020), was used to test whether a particular algal species can exert the post-illumination fluorescence rise. Activated F_0 rise was quantified by integrating the fluorescence signal during 60 s of the post-illumination period (fluorescence values lower than those at the moment when the actinic illumination ended, were subtracted).

F_0 rise measurements – traditional F_0 rise: In some cases, as indicated, F_0 rise was measured with the 'original' protocol, with a *Multi-Color PAM* (*MC-PAM*; *Heinz Walz GmbH*). Blue (480 nm, PPFD ~2.5 µmol m^{-2} s^{-1}) ML of *MC-PAM* functioned as the activating light, and was on for the whole measurement period, with the frequency of 5 kHz (before and after the pre-illumination) or 20 kHz (during the pre-illumination). White actinic light (PPFD of 50 µmol m^{-2} s^{-1}) or far-red light (for the spectra, *see* Fig. 1S) of the *MC-PAM* was used as the pre-illumination, as indicated. Activated F_0 rise was quantified by integrating the fluorescence signal during 100 s of the post-illumination period. In one set of experiments, 1 mM propyl gallate, an inhibitor of PTOX, was added to the (liquid) algal culture 5 min before the fluorescence measurement started. Otherwise, the conditions were identical to those of the activated F_0 rise protocol described above.

P_{700} measurements: Simultaneous measurements of fluorescence and P_{700} absorbance signal were conducted with *Dual KLAS NIR*, with the *ED-101US/MD* accessory cuvette for liquid measurements (*Heinz Walz GmbH*). Since no calibration was available for green algae, only the P_{700} signal and fluorescence were measured. The cuvette temperature was set to 26°C, blue ML was used and far-red was set to maximum. After control measurements, oxygen was removed by adding 6 mM glucose, 800 U ml^{-1} glucose oxidase, and 8 U ml^{-1} catalase (*Sigma Aldrich*), and the sample was incubated for 15 min at room temperature in the dark after which the measurement was repeated.

Statistics: Statistical differences were tested by calculating the *Student*'s *t*-test (heteroscedastic) in *Microsoft Excel*, based on at least three independent replicates. Asterisks ***, **, and * indicate a probability of $P<0.001$, $P<0.01$, and $P<0.05$, respectively, of the null hypothesis.

Results

Application of the F_0 rise protocol to green microalgae: We recorded the post-illumination fluorescence rise (F_0 rise) using 660 and 700-nm weak activating light for preferential excitation of PSII or PSI, respectively (for the protocol, *see* Fig. 2S). The method was originally designed to screen the photosystem preference of a visible light wavelength in higher plants (Mattila *et al*. 2020). Here, preliminary measurements in six green algal species revealed that three *Scenedesmus* species, namely *S. quadricauda*, *S. ecornis*, and *S. fuscus*, showed a rise in F_0 fluorescence level under 660-nm activating light and, like plants, lacked an F_0 rise at 700-nm activating light (Table 1). Examples of the fluorescence traces from *S. quadricauda* are shown in Fig. 1; switching off the white pre-illumination and switching on the weak 660-nm activating light caused a sharp, ~70% increase in the fluorescence level during the first 5 s, followed by a slow decline to the initial fluorescence level. When activating light of 700 nm was applied, no post-illumination fluorescence rise was observed.

E. oleoabundans, in turn, showed almost no F_0 rise in response to 660 nm, nor to 700 nm activating light. *C. reinhardtii* and *C. sorokiniana* exhibited differences

Table 1. Occurrence of the F_0 rise in different green algae species.

Species	Strains	Protocol	F_0 rise pattern	Cultivation conditions	Medium
Scenedesmus quadricauda		660-nm activating light, *PAM-101*	Plant-like	A, B, C	BG11
Scenedesmus ecornis		660-nm activating light, *PAM-101*	Plant-like	C	BG11
Scenedesmus fuscus		660-nm activating light, *PAM-101*	Plant-like	C	BG11
		480-nm ML, *MC-PAM*	Plant-like	B	
Chlorella sorokiniana		660-nm activating light, *PAM-101*	Unobvious	A, C	BG11
Chlamydomonas reinhardtii	125 (137C)	660-nm activating light, *PAM-101*	Unobvious	A, C	HSM
	cc4533	480-nm ML, *MC-PAM*	Plant-like	B	
	flvB (cc4533)	480-nm ML, *MC-PAM*	Unobvious	B	
Ettlia oleoabundans		660-nm activating light, *PAM-101*	Unobvious	A, C	BG11
		480-nm ML, *MC-PAM*	Unobvious	B	

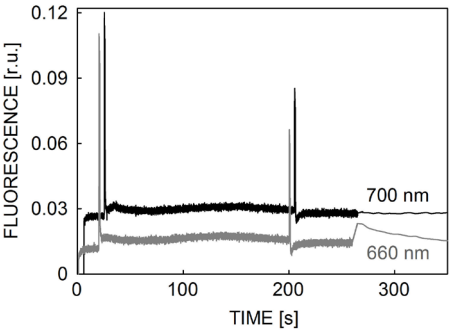

Fig. 1. Chlorophyll *a* fluorescence traces measured from *Scenedesmus quadricauda*, grown under conditions A (semi-continuous, moderate light). White light from a *KL-1500* illuminator was used as the pre-illumination (PPFD of 50 µmol m^{-2} s^{-1}). The wavelengths of the monochromatic activating light (PPFD of 2.5 µmol m^{-2} s^{-1}) were 660 nm (*grey trace*) and 700 nm (*black trace*). The 700-nm curve has been shifted upwards and to the right for clarity. Representative curves are shown.

between activated F_0 rise traces measured with 660 and 700-nm activating light, although a clear transient increase in the fluorescence yield after the switch-off of the pre-illumination was absent (Table 1).

Exposure of *S. quadricauda* cells to the white pre-illumination (PPFD of 50 µmol m^{-2} s^{-1}) for 3 min induced moderate NPQ as indicated by the finding that the F_M' level was about 20% lower than F_M (Fig. 1). This level of NPQ, even if it partially relaxes during the monitoring of the F_0 rise, would only have a minor influence on the F_0 rise transient.

Action spectra of activated F_0 rise in *S. quadricauda* and *S. fuscus*: Since *S. quadricauda* expressed a clear F_0 rise pattern, we used this species to measure the action spectra of the F_0 rise. *S. quadricauda* was grown under three different growth conditions (semi-continuous growth under moderate light, and batch growth under high or low light, indicated by the letters A, B, and C, respectively; *see* 'Materials and methods' for details). *S. quadricauda* showed a high F_0 rise at activating light wavelengths of 470–490 nm (blue peak), 650–660 nm (red peak), and 560–600 nm (broad green peak), whereas 420–450 nm (blue deep), 520–530 nm (green deep), 620–630 nm (orange deep), and 680–690 nm (red deep) activating light exhibited a low capacity to generate an F_0 rise (Fig. 2*A–C*). Such features are also observed in an F_0 spectrum measured from tobacco leaves (Mattila *et al.* 2020). The amplitude of the F_0 rise was higher in the alga than that in the plant. However, optical differences between plant leaves and algal cells may have contributed to the difference. Furthermore, the action spectra were similar in *S. quadricauda* grown under the three different growth conditions; growth conditions appeared to mostly affect the shape and maximum values of the broad green peak (560–600 nm), which did not have a consistent maximum in the three conditions. The maximal amplitudes of the activated F_0 rise were also higher in cells grown under high light (condition B) and the red peak (660–670 nm) was shifted to the right, compared to the other conditions. The LED illumination used in condition B (*see* Fig. 1S for the spectra) contained more red and far-red light than the fluorescent light used for other growth conditions.

The action spectrum of the F_0 rise was also measured from *S. fuscus* (grown under condition C), with a lower resolution. The spectrum had the same peaks and deeps as that of *S. quadricauda* (Fig. 2*D*).

Effect of PTOX activity on F_0 rise: To get more insights into the fact that not all algal species showed an F_0 rise (Table 1), we repeated the F_0 rise measurement in the presence of propyl gallate, a PTOX inhibitor, with *S. fuscus* (an alga showing a clear F_0 rise) and with *E. oleoabundans* (an alga showing no F_0 rise). In this case, we used the 'original' F_0 rise protocol, where the weak measuring light of the fluorometer (480 nm in this case) is continuously on during the F_0 rise measurement and acts as the activating light. *S. fuscus* showed again a clear F_0 rise, but also *E. oleoabundans* showed a tiny F_0 rise (Fig. 3). The addition of propyl gallate increased the size of the F_0 rise in both algae, even though *E. oleoabundans* still showed a very small F_0 rise (Fig. 3). The addition of propyl gallate

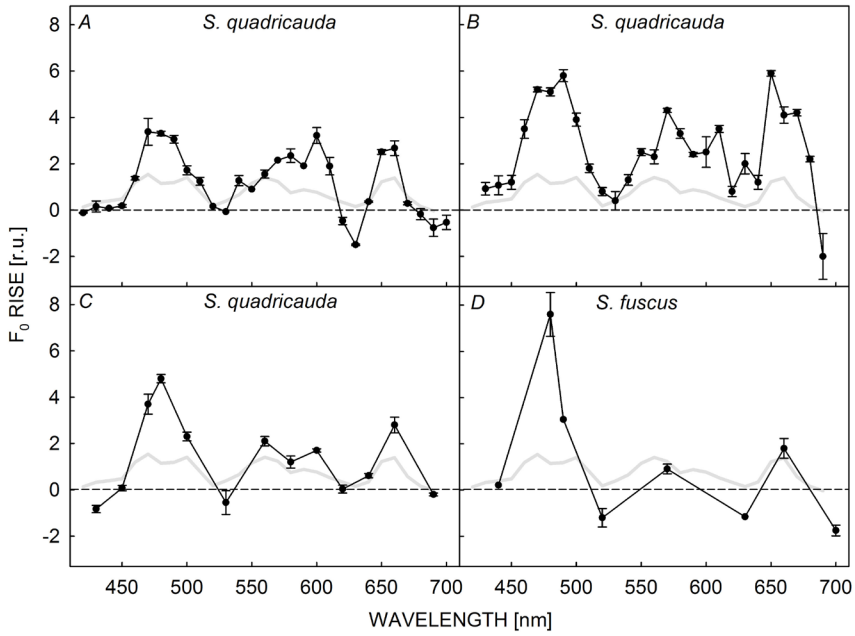

Fig. 2. Action spectra of wavelength-activated F_0 rise in *Scenedesmus quadricauda* (*A–C*) cells cultivated under conditions A (semi-continuous, moderate light; *A*), B (batch, high light; *B*) or C (batch, low light; *C*) and *Scenedesmus fuscus* (*D*) cells cultivated under conditions C. White light from a *KL-1500* illuminator was used as the pre-illumination (PPFD of 50 µmol m^{-2} s^{-1}). The *x*-axis indicates the wavelength of the monochromatic activating light (PPFD of 2.5 µmol m^{-2} s^{-1}). Data represent the mean of three replicates and standard error. *Light gray lines* in the background show similar spectra from tobacco (Mattila *et al*. 2020), for comparison.

increased also the amount of NPQ, especially in *S. fuscus*, and therefore, the measurements were also performed with a dimmer pre-illumination (PPFD of 35 µmol m^{-2} s^{-1}) for *S. fuscus* and with a far-red light (*see* Fig. 1S for the light spectrum) as the pre-illumination for both *S. fuscus* and *E. oleoabundans*. Lowering the pre-illumination intensity reduced NPQ formation (Fig. 3*A*), and after the far-red pre-illumination, no NPQ developed (Fig. 3*A*,*B*). Far-red pre-illumination increased the size of the F_0 rise in both algae, but the increase was significant only in *E. oleoabundans*. The addition of propyl gallate further increased the size of the F_0 rise only in *E. oleoabundans* (Fig. 3). In addition, in the case of *S. fuscus* but not of *E. oleoabundans*, propyl gallate addition slowed down the F_0 fluorescence decrease after the F_0 rise, in all the treatments (Fig. 3*A*,*B*).

F_0 rise in *C. reinhardtii*: As indicated in Table 1, *C. reinhardtii* [the 125 (137C) wild type] did not show a clear F_0 rise. However, fluorescence traces measured with 660 and 700 nm activating light showed obvious differences; with 660 nm activating light, the fluorescence yield declined gradually to a stable minimal level in 20 s while the decrease of fluorescence yield was rapid when 700 nm activating light was used (Fig. 4*A*).

The fluorescence traces of *C. reinhardtii* resembled those measured from *Arabidopsis*, when using a pre-illumination that reduces rather than oxidizes the PQ pool (Mattila *et al*. 2020). Therefore, the measurement protocol was modified so that 690 nm light (PPFD of 50 µmol m^{-2} s^{-1}) was used as the pre-illumination for efficient oxidation of the PQ pool. Furthermore, blue wavelengths of 420 nm (PSI light) and 470 nm (PSII light) were used as activating lights because 470 nm actinic light induced a slightly stronger activated F_0 rise in *S. quadricauda* than that of 660 nm activating light (Fig. 2). The ML cycles for estimating F_0 rise were also applied at a higher frequency (0.6 s on/1 s off). Application of 420 nm PSI light as the activating light caused a rapid (1 s) drop in fluorescence yield after switching off the 690 nm pre-illumination, and the fluorescence level remained unaltered until the end of the measurement (Fig. 4*B*). When 470 nm activating light (PSII light) was applied, fluorescence initially declined for 1 s, and thereafter increased for 5 s, and then gradually decreased to a minimum (Fig. 4*B*). It seems, therefore, that the white light from *KL-1500* indeed did not oxidize the PQ pool in *C. reinhardtii* 125 (137C). The F_0 rise after the 690 nm pre-illumination was still quite small.

To further understand the conditions determining the size of the F_0 rise in *C. reinhardtii*, we used a mutant lacking the FlvB protein (Jokel *et al*. 2018) and its corresponding wild type [cc4533, a different strain from the above used *C. reinhardtii* 125 (137C)]. Here we used the white or far-red light of the *MC-PAM* fluorometer, as indicated, as the pre-illumination light while the weak 480 nm measuring light was used as the activating light. In contrast to the *C. reinhardtii* 125 (137C), the cc4533 strain produced a strong F_0 rise, under both types of the pre-illumination light (Fig. 5). The deletion of the FlvB, in turn, clearly diminished the size of the F_0 rise (Fig. 5).

Effect of oxygen removal on P_{700} kinetics in *S. fuscus* and *E. oleoabundans*: To test if flavodiiron activity could explain the varying sizes of the F_0 rise also in the other green algal species, we measured the P_{700} (the primary electron donor of PSI) oxidation capacity of *S. fuscus* and *E. oleoabundans* in the presence and absence of oxygen. In the case of *S. fuscus*, far-red and high-light pulses were able to reduce P_{700} in the presence of oxygen, but when oxygen was removed, only a transient spike upon the high-light pulse was observed (Fig. 6*A*,*B*). P_{700} oxidation pattern in *E. oleoabundans*, in turn, was not affected by oxygen removal (Fig. 6*C*,*D*).

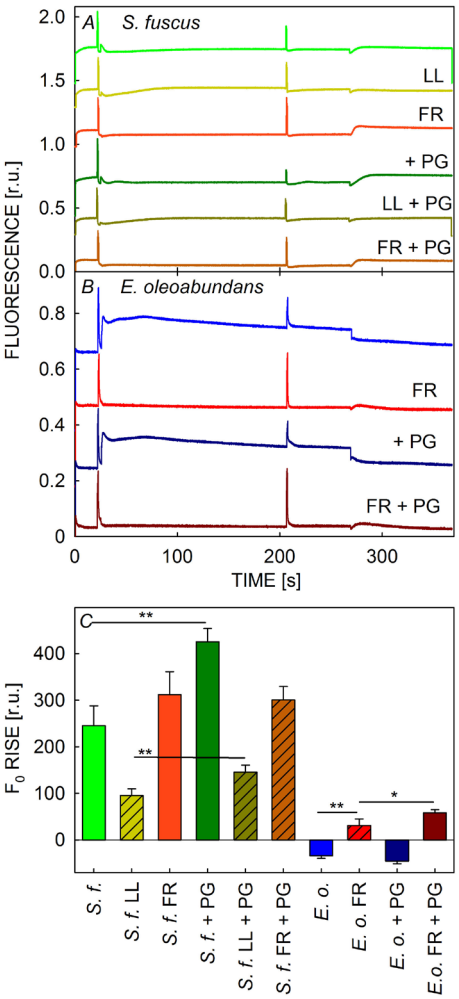

Fig. 3. Fluorescence traces (*A*,*B*) and quantification of the F_0 rise (*C*) in *Scenedesmus fuscus* and *Ettlia oleoabundans*, grown under conditions B (batch, high light). White or far-red (FR) light from *MC-PAM* was used as the pre-illumination [PPFD of 50 or 35 (LL) µmol m^{-2} s^{-1}], as indicated. The 480-nm measuring beam (PPFD ~2.5 µmol m^{-2} s^{-1}) was used as the activating light. The measurement was conducted either in the absence or presence of propyl gallate (PG). The fluorescence traces in (*A*,*B*) have been shifted for clarity. Representative curves are shown. Each bar in (*C*) represents the mean of three replicates and the error bars show SD. *The asterisks* indicate a statistical significance, estimated with the *t*-test, between the indicated groups.

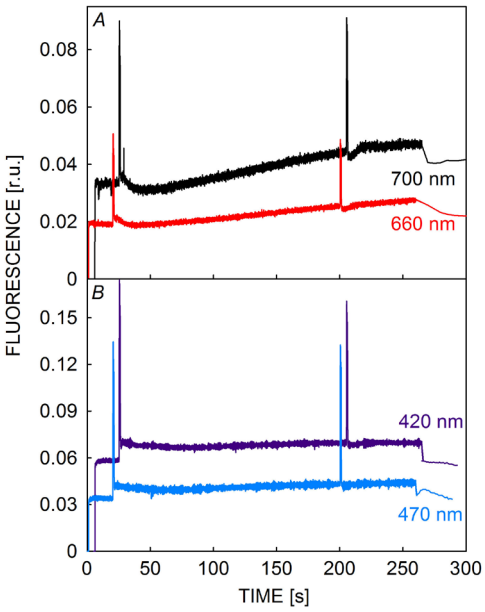

Fig. 4. Chlorophyll *a* fluorescence traces of *Chlamydomonas reinhardtii* 125 (137C), grown under conditions A (semi-continuous, medium light), measured using white from a *KL-1500* illuminator (*A*) or 690 nm (*B*) as pre-illumination (PPFD of 50 µmol m^{-2} s^{-1}). The wavelengths of the monochromatic activating light (PPFD of 2.5 µmol m^{-2} s^{-1}) were 700, 660, 420, or 470 nm, as indicated. The ML cycle for estimating F_0 rise was 0.6 s on/5 s off in panel A and 0.6 s on/1 s off in panel *B*. The 700 (*A*) and 420 (*B*) nm curves have been shifted upwards and to the right for clarity. Representative curves are shown.

Discussion

In higher plants, the wavelengths causing a high F_0 rise were shown to favor PSII over PSI, and also to reduce the PQ pool at a moderate intensity. A high activated F_0 rise was obtained with 460–500 nm (blue peak), 560 nm (green peak), and 650–660 nm (red peak) activating light, whereas the F_0 rise was weak or missing at 420–450 nm (blue deep), 520 nm (green deep), 630 nm (orange deep), and 680–690 nm (red deep) lights. It was concluded that the form of the action spectrum of the F_0 rise is mainly determined by the ratio of Chl *b* to Chl *a* in the antenna complexes (Mattila *et al.* 2020). In the present study, a similar F_0 rise protocol was applied to several green algal species but only *Scenedesmus* species (*S. quadricauda*, *S. ecornis*, *S. fuscus*) exhibited an explicit plant-like F_0 rise. The F_0 rise spectra of *S. quadricauda* (Fig. 2) were highly similar to that of tobacco (Mattila *et al.* 2020), suggesting that the spectral properties of the two photosystems are very conserved between land plants and green algae (Viridiplantae), even though algae are characterized by peculiar structural organizations of peripheral antenna complexes (for a review, *see* Rochaix 2014). Also, the redox state of the PQ pool, after illumination with several wavelengths favoring either PSII or PSI, shows a similar response in *Arabidopsis* and *C. reinhardtii* (Mattila *et al.* 2020, Virtanen and Tyystjärvi 2022). However, more green algal species should be investigated.

The action spectra of the F_0 rise of *S. quadricauda* were very similar after cultivation of the algae in three different experimental conditions differing in light quantity and quality (Figs. 1S, 2S). Interestingly, though, 670–680 nm light produced a relatively high F_0 rise only when *S. quadricauda* was grown under conditions B, where the growth light had significant red and far-red contribu-

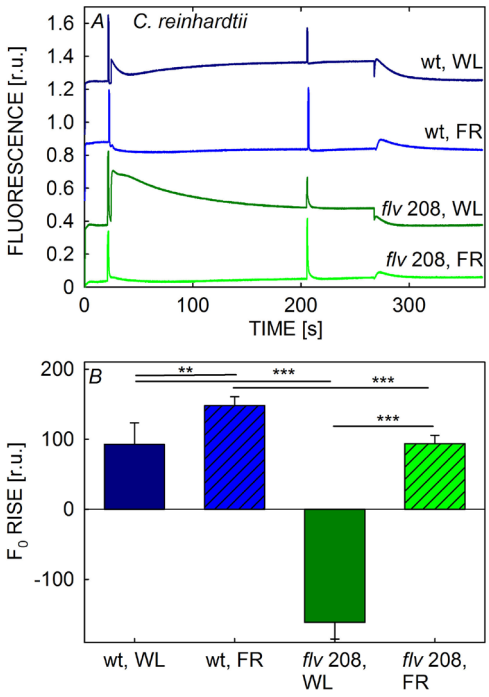

Fig. 5. Fluorescence traces (*A*) and quantification of the F_0 rise (*B*) in *Chlamydomonas reinhardtii* (cc4533) wild type (wt), and FlvB deletion mutant (*flv* 208), grown under conditions B (batch, high light). White (WL) or far-red (FR) light from an *MC-PAM* was used as the pre-illumination (PPFD of 50 µmol m^{-2} s^{-1}), as indicated. A 480-nm measuring light (PPFD ~2.5 µmol m^{-2} s^{-1}) was used as the activating light. The fluorescence traces in (*A*) have been shifted for clarity. Representative curves are shown. Data in (*B*) represent the mean of three replicates and the standard error. *The asterisks* indicate statistical significance between the indicated groups.

tions, unlike in the other growth conditions. This might suggest that the growth under red and far-red enriched light modified absorption properties of the antennae in *S. quadricauda*, possibly due to state transitions and/or other modifications of antennae composition and structure (Ueno *et al*. 2019, Wolf and Blankenship 2019). The observation that higher values of F_0 rise were observed after growth under high light (condition B; Fig. 2) may suggest a better capacity of these cells to keep the PQ pool oxidized during the pre-illumination.

In plant leaves, the F_0 rise method can be used, at most wavelengths, to predict the capacity of the wavelength to reduce/oxidize the PQ pool (Mattila *et al*. 2020). However, the 630 nm orange deep (*i.e.*, a PSI light) is an exception, as the *A. thaliana* PQ pool was found to remain ~50% reduced at this wavelength. The finding that the activated F_0 rise shows a clear orange deep also in *S. quadricauda* (Fig. 2) suggests that 630 nm light truly favors PSI.

Unlike the *Scenedesmus* species, *C. reinhardtii* [the 125 (137C) strain], *C. sorokiniana*, and *E. oleoabundans* did not exhibit a clear F_0 rise (with a PSII-activating light) when white pre-illumination was used (Table 1; Figs. 3, 4). The size of the F_0 rise can be modulated by four factors: (*1*) the oxidation state of the PQ pool at the end of the pre-illumination, as it was shown that a big F_0 bump only occurs if the pre-illumination oxidizes the PQ pool (Mattila *et al*. 2020), (*2*) the PSII/PSI nature of the activating light, (*3*) the rate of the reduction of the PQ pool after switching off the pre-illumination, which in *A. thaliana* was shown to occur *via* the activity of the chloroplast NADH dehydrogenase-like (NDH) complex (Mattila *et al*. 2020), and (*4*), theoretically, by the rate of the oxidation of the PQ pool after switching off the pre-illumination. A clear, although small F_0 rise could then be seen in *C. reinhardtii* [the 125 (137C) strain] and *E. oleoabundans* by using far-red light (a clear PSI light) as the pre-illumination

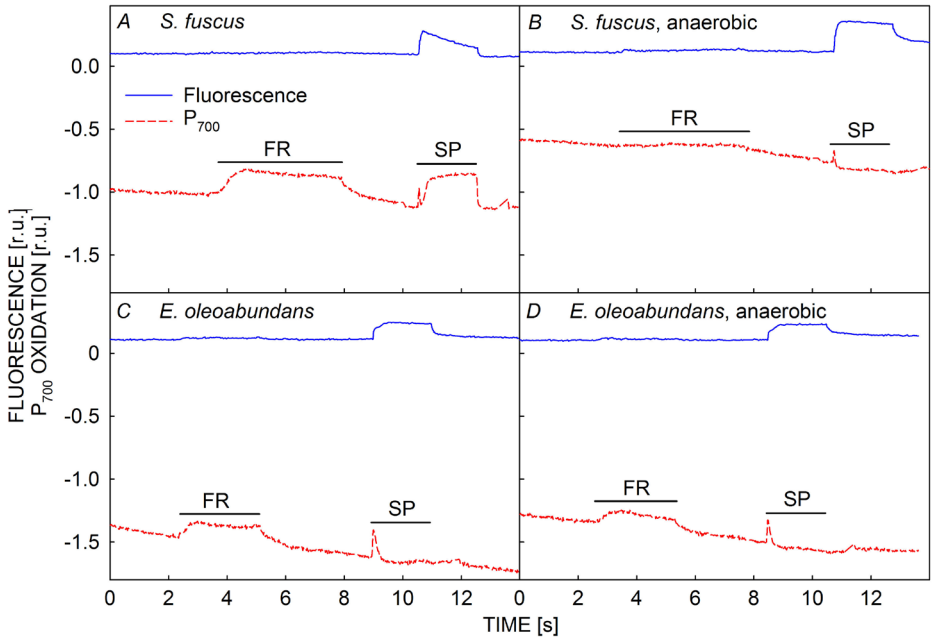

Fig. 6. Fluorescence and P_{700} traces in *Scenedesmus fuscus* (*A*,*B*) and *Ettlia oleoabundans* (*C*,*D*), during aerobic (*A*,*C*) and anaerobic (*B*,*D*) conditions. FR indicates a far-red light and SP a saturating pulse. The algae were grown under conditions B (batch, high light). Representative curves are shown.

(Figs. 3, 4), indicating that the white light illumination with a *KL-1500* illuminator did not oxidize the PQ pool in these algae. In contrast, in *A. thaliana*, the *KL-1500* tends to oxidize the PQ pool (Mattila *et al*. 2020), as well as presumably also in the *Scenedesmus* species used here.

In the other *C. reinhardtii* strain (cc4533), flavodiiron proteins seemed to contribute to the oxidation of the PQ pool during white light pre-illumination, as an absence of the FlvB and FlvA proteins (Jokel *et al*. 2018) decreased the amplitude of the F_0 rise (Fig. 5). The explanation is supported by the observation that in the *flvB* mutant fluorescence yield stayed at a higher level during the pre-illumination, compared to the wild type (Fig. 5*A*). Large effects of the flavodiiron proteins on chlorophyll fluorescence phenomena in *C. reinhardtii* have been demonstrated also earlier (Jokel *et al*. 2018). Intriguingly, the two *C. reinhardtii* strains used showed very different F_0 rise patterns (Figs. 4, 5), suggesting different strategies for balancing the electron transfer chain. However, we cannot rule out the possibility that different growth conditions or F_0 protocols could have contributed to the differences.

Whether flavodiiron proteins are important also in other algae in keeping the PQ pool oxidized during a white light illumination is difficult to judge as the presence of flavodiiron proteins in the studied species (except for *C. reinhardtii*) is not known. *Scenedesmus* shows light-dependent oxygen reduction (Radmer and Kok 1976), which has been interpreted to indicate the presence of flavodiiron proteins (*e.g.*, Peltier *et al*. 2010), but BLAST searches for genes coding for proteins related to *Chlamydomonas* FlvA or FlvB proteins did not yield significant homologs in *Scenedesmus* species. BLAST searches with the same proteins did reveal significant homologs in *Chlorella ohadii* and *Chlorella variabilis*, indicating that flavodiiron proteins can also be found in Treubouxiophyceae (incl. *Chlorella*). As flavodiiron proteins donate electrons to oxygen, the sensitivity of P_{700} kinetics to anaerobiosis has been previously used to screen flavodiiron-like activity in various algal species (Shimakawa *et al*. 2019). P_{700} oxidation kinetics in *E. oleoabundans* (no or very small F_0 rise) were unaffected by the removal of oxygen, in contrast to *S. fuscus* (big F_0 rise) where P_{700} oxidation was disturbed in the absence of oxygen (Fig. 6). It can be hypothesized, then, that in those species with a high F_0 rise, the PQ pool remained at a more oxidized state during a white light pre-illumination, due to a high flavodiiron activity.

Even with the far-red pre-illumination the F_0 rise was very small in the 125 (137C) strain of *C. reinhardtii* and in *E. oleoabundans* (Figs. 3, 4), suggesting that the difficulties to keep the PQ pool oxidized during a white pre-illumination were not the only reason for a small F_0 rise, but either the post-illumination reduction of the PQ pool was slow, or its concomitant re-oxidation was fast. PTOX is the only known mechanism that oxidizes the (thylakoid) PQ pool in the dark. Indeed, the *ptox2* mutant of *C. reinhardtii* has earlier been shown to possess a high F_0 rise (Houille-Vernes *et al*. 2011). The addition of propyl gallate (a PTOX inhibitor) did increase the size of the F_0 rise in *E. oleoabundans* and *S. fuscus*, and also the disappearance of the F_0 bump was hindered in *S. fuscus* (Fig. 3). However, the changes were small, suggesting that the rate of the PQ oxidation by PTOX was slow in the studied algae.

In *C. reinhardtii* cc4533 and *E. oleoabundans*, the F_0 rise level after the F_0 bump decreased slower after far-red pre-illumination than after white pre-illumination (Figs. 3, 5). Changes in NPQ relaxation or the rate of PQ reduction in the dark may explain these observations, rather than differences in PTOX activity, as the same trend was also observed with propyl gallate in *E. oleoabundans* (Fig. 3*B*).

In green algae and plants, the PGR5/PGRL1 and NDH-1 or NDH-2 mediated pathways are known contributors to the nonphotochemical (dark) reduction of the PQ pool (for a review, *see* Peltier *et al*. 2016), and consequently to the F_0 rise. Electron transfer through the PGR5/PGRL1-dependent route is only weakly associated with the F_0 rise in plants (Munekage *et al*. 2002, Nellaepalli *et al*. 2015) whereas F_0 rise is suppressed in the NDH-1 deficient *ndh-0* mutant of *A. thaliana* (Shikanai *et al*. 1998, Mattila *et al*. 2020). Algae belonging to classes Chlorophyceae (*Chlamydomonas*, *Scenedesmus*, *Ettlia*) and Treubouxiophyceae (*Chlorella*) have lost chloroplastic *ndh* genes (Peltier *et al*. 2016). Nevertheless, in *C. reinhardtii*, the nucleus-encoded Nda2, a type II NAD(P)H dehydrogenase (NDH-2), mediates electron transfer from NAD(P)H to PQ (Desplats *et al*. 2009). The presence of an F_0 rise in *C. reinhardtii* (Figs. 4, 5) suggests that NDH-2 activity, too, can support an F_0 rise phenomenon. A BLAST search for sequences producing significant alignments with Nda2 of *C. reinhardtii* (EDO96450.1) revealed five proteins in *Chlorella variabilis* with 32, 41, 45, 49, and 58% similarity but no significant similarity was found in *Tetradesmus obliquus* (former *Scenedesmus obliquus*). Thus, the presence of NDH-2 (and consequently the mechanism of F_0 rise formation) in the species used in the present study, except for *C. reinhardtii*, is not known. Anyway, the presence of an F_0 rise in the studied *Scenedesmus*, *Chlorella*, and *Tetradesmus* species suggests that these species, too, have a pathway by which ferredoxin or NADPH reduces the PQ pool.

The dynamics of the stromal electron donors during the post-illumination period may also explain differences in sizes and patterns of the F_0 rise between different algal species or different growth conditions. Reduction of $NADP^+$ depends on reduced ferredoxin, and therefore rapid oxidation of NADPH either by the remaining activity of the Calvin–Benson cycle, stromal flavodiiron proteins, or Mehler's reaction would lower the F_0 rise irrespective of whether reduction of PQ depends on ferredoxin or NADPH as the electron donor. It is also possible that PSI and PSII antennas in those algae showing a small F_0 rise have relatively similar absorption properties, and therefore visible light wavelengths do not as clearly favor one photosystem over the other.

In conclusion, the findings of our work show that the spectral distribution of PSII and PSI excitation can be very similar between land plants and green algae. However,

we showed that the magnitude and duration of the F_0 rise vary significantly among algae species. This diversity suggests variation in the balance between reduction and oxidation of the PQ pool during both the pre-illumination and the post-illumination period; flavodiiron proteins appear to be important during illumination while PTOX has a minor role after the light has been switched off. The F_0 rise method can be employed for screening alternative electron transport pathways in green algae species as well as in mutants. However, such implementation requires further exploration of the mechanisms involved in the formation of the F_0 rise in green algae.

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