



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

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### 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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**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 CICALA (<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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . 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<sub>730</sub> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in an *Algaetron* (PSI, Czech Republic). The measurements were done after 7 d of cultivation after which OD<sub>730</sub> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The measurements were done after 14 d of cultivation after which OD<sub>730</sub> 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<sub>0</sub> rise measurements – activated F<sub>0</sub> rise:** Chl *a* fluorescence was measured with a *PAM-101* fluorometer (*Heinz Walz GmbH*, Germany) according to the activated F<sub>0</sub> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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<sup>-2</sup>; Chl was extracted by at least 24-h incubation in methanol at 4°C in the dark (*Inskip 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 0.8 s) was fired and an actinic light (PPFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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<sub>0</sub> 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), 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<sub>0</sub> 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<sub>0</sub> rise measurements – traditional F<sub>0</sub> rise:** In some cases, as indicated, F<sub>0</sub> rise was measured with the ‘original’

protocol, with a *Multi-Color PAM* (*MC-PAM*; *Heinz Walz GmbH*). Blue (480 nm, PPFD ~2.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) or far-red light (for the spectra, *see* Fig. 1S) of the *MC-PAM* was used as the pre-illumination, as indicated. Activated F<sub>0</sub> 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<sub>0</sub> rise protocol described above.

**P<sub>700</sub> measurements:** Simultaneous measurements of fluorescence and P<sub>700</sub> 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<sub>700</sub> 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<sup>-1</sup> glucose oxidase, and 8 U ml<sup>-1</sup> 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<sub>0</sub> rise protocol to green microalgae:** We recorded the post-illumination fluorescence rise (F<sub>0</sub> 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<sub>0</sub> fluorescence level under 660-nm activating light and, like plants, lacked an F<sub>0</sub> 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<sub>0</sub> 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
<i>Scenedesmus quadricauda</i>		660-nm activating light, <i>PAM-101</i>	Plant-like	A, B, C	BG11
<i>Scenedesmus ecornis</i>		660-nm activating light, <i>PAM-101</i>	Plant-like	C	BG11
<i>Scenedesmus fuscus</i>		660-nm activating light, <i>PAM-101</i>	Plant-like	C	BG11
		480-nm ML, <i>MC-PAM</i>	Plant-like	B	
<i>Chlorella sorokiniana</i>		660-nm activating light, <i>PAM-101</i>	Unobvious	A, C	BG11
<i>Chlamydomonas reinhardtii</i>	125 (137C)	660-nm activating light, <i>PAM-101</i>	Unobvious	A, C	HSM
	cc4533	480-nm ML, <i>MC-PAM</i>	Plant-like	B	
	<i>flvB</i> (cc4533)	480-nm ML, <i>MC-PAM</i>	Unobvious	B	
<i>Ettlia oleoabundans</i>		660-nm activating light, <i>PAM-101</i>	Unobvious	A, C	BG11
		480-nm ML, <i>MC-PAM</i>	Unobvious	B	

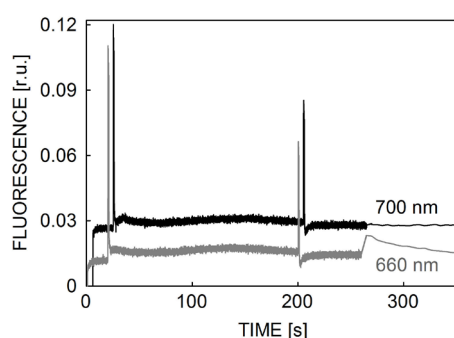


Fig. 1. Chlorophyll *a* fluorescence traces measured from *Scenedesmus quadricauda*, grown under conditions A (semi-continuous, moderate light). White light from a *KL-1500* illuminator was used as the pre-illumination (PPFD of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The wavelengths of the monochromatic activating light (PPFD of  $2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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. 2A–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. 2D).

**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

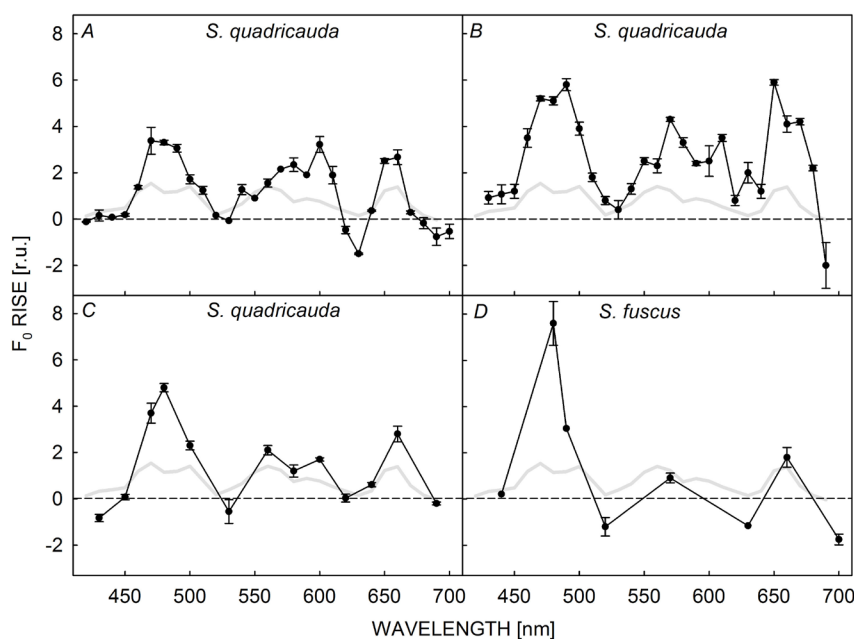


Fig. 2. Action spectra of wavelength-activated F<sub>0</sub> rise in *Scenedesmus quadricauda* (A–C) cells cultivated under conditions A (semi-continuous, moderate light; A), B (batch, high light; B) or C (batch, low light; C) and *Scenedesmus fuscus* (D) cells cultivated under conditions C. White light from a KL-1500 illuminator was used as the pre-illumination (PPFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). The x-axis indicates the wavelength of the monochromatic activating light (PPFD of 2.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). 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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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. 3A), and after the far-red pre-illumination, no NPQ developed (Fig. 3A,B). Far-red pre-illumination increased the size of the F<sub>0</sub> 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<sub>0</sub> 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<sub>0</sub> fluorescence decrease after the F<sub>0</sub> rise, in all the treatments (Fig. 3A,B).

**F<sub>0</sub> rise in *C. reinhardtii*:** As indicated in Table 1, *C. reinhardtii* [the 125 (137C) wild type] did not show a clear F<sub>0</sub> 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. 4A).

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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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<sub>0</sub> rise in *S. quadricauda* than that of 660 nm activating light (Fig. 2). The ML cycles for estimating F<sub>0</sub> 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. 4B). 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. 4B). It seems, therefore, that the white light from KL-1500 indeed did not oxidize the PQ pool in *C. reinhardtii* 125 (137C). The F<sub>0</sub> rise after the 690 nm pre-illumination was still quite small.

To further understand the conditions determining the size of the F<sub>0</sub> 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<sub>0</sub> 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<sub>0</sub> rise (Fig. 5).

**Effect of oxygen removal on P<sub>700</sub> kinetics in *S. fuscus* and *E. oleoabundans*:** To test if flavodiiron activity could explain the varying sizes of the F<sub>0</sub> rise also in the other green algal species, we measured the P<sub>700</sub> (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<sub>700</sub> in the presence of oxygen, but when oxygen was removed, only a transient spike upon the high-light pulse was observed (Fig. 6A,B). P<sub>700</sub> oxidation pattern in *E. oleoabundans*, in turn, was not affected by oxygen removal (Fig. 6C,D).

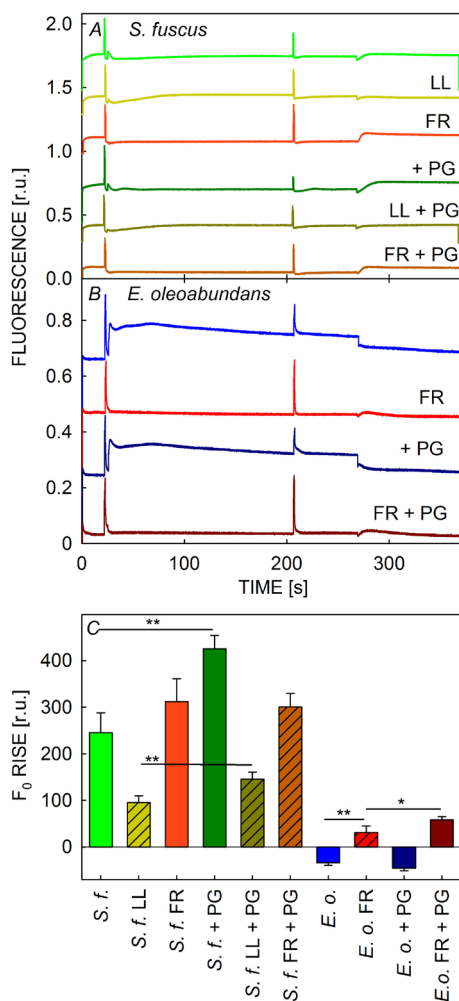


Fig. 3. Fluorescence traces (A,B) and quantification of the  $F_0$  rise (C) in *Scenedesmus fuscus* and *Ettlia oleoabundans*, grown under conditions B (batch, high light). White or far-red (FR) light from *MC-PAM* was used as the pre-illumination [PPFD of 50 or 35 (LL)  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ], as indicated. The 480-nm measuring beam (PPFD  $\sim 2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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.

## 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

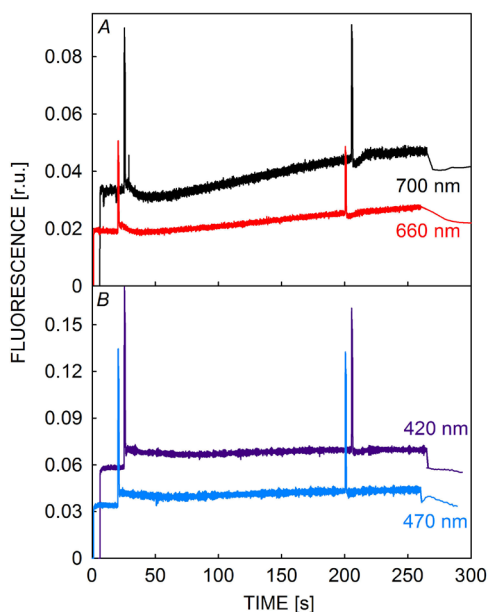


Fig. 4. Chlorophyll *a* fluorescence traces of *Chlamydomonas reinhardtii* 125 (137C), grown under conditions A (semi-continuous, medium light), measured using white from a *KL-1500* illuminator (A) or 690 nm (B) as pre-illumination (PPFD of  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The wavelengths of the monochromatic activating light (PPFD of  $2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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.

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-

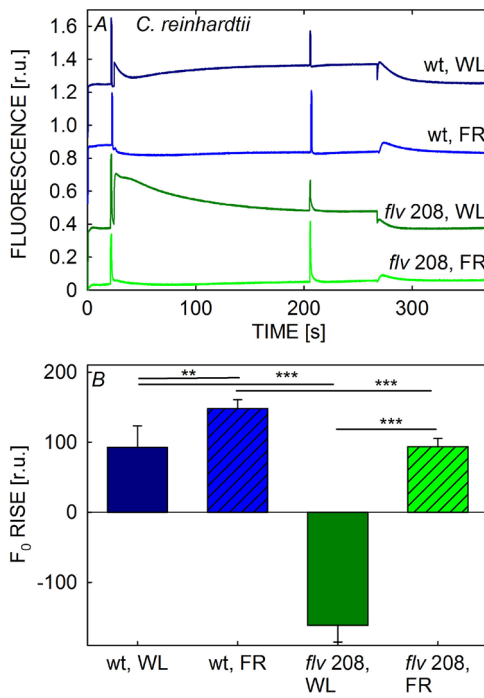


Fig. 5. Fluorescence traces (A) and quantification of the F<sub>0</sub> rise (B) in *Chlamydomonas reinhardtii* (cc4533) wild type (wt), and FlvB deletion mutant (*flv 208*), grown under conditions B (batch, high light). White (WL) or far-red (FR) light from an MC-PAM was used as the pre-illumination (PPFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), as indicated. A 480-nm measuring light (PPFD  $\sim 2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) 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<sub>0</sub> 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<sub>0</sub> 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 PSII light) is an exception, as the *A. thaliana* PQ pool was found to remain  $\sim 50\%$  reduced at this wavelength. The finding that the activated F<sub>0</sub> 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<sub>0</sub> rise (with a PSII-activating light) when white pre-illumination was used (Table 1; Figs. 3, 4). The size of the F<sub>0</sub> 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<sub>0</sub> 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<sub>0</sub> 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

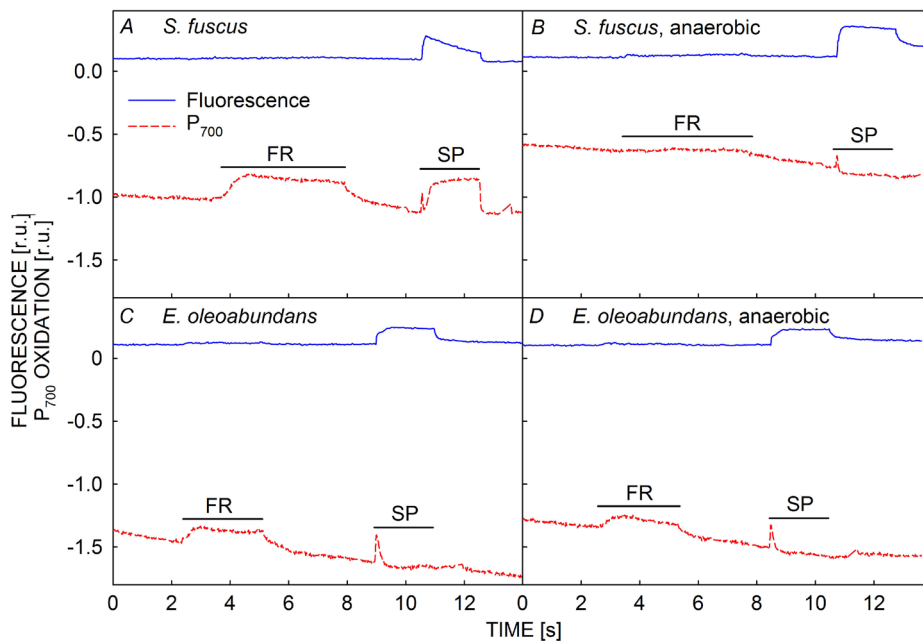


Fig. 6. Fluorescence and P<sub>700</sub> traces in *Scenedesmus fuscus* (A,B) and *Ectliia oleoabundans* (C,D), during aerobic (A,C) and anaerobic (B,D) conditions. FR indicates a far-red light and SP a saturating pulse. The algae were grown under conditions B (batch, high light). Representative curves 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. 5A). 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. 3B).

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<sup>+</sup> 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<sub>0</sub> 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<sub>0</sub> 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<sub>0</sub> rise in green algae.

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