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BRIEF COMMUNICATION

Effects of different excitation and detection spectral regions on room temperature chlorophyll *a* fluorescence parameters

M. ŠTROCH[*], J. PODOLINSKÁ, M. NAVRÁTIL[*], and V. ŠPUNDA[*,**,***]

Department of Physics, Faculty of Science, Ostrava University, 30. dubna 22, 701 03 Ostrava 1, Czech Republic[*]
Institute of Physical Biology, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic[**]

Abstract

The effects of different spectral region of excitation and detection of chlorophyll (Chl) *a* fluorescence at room temperature on the estimation of excitation energy utilization within photosystem (PS) 2 were studied in wild-type barley (*Hordeum vulgare* L. cv. Bonus) and its Chl *b*-less mutant *chlorina f2* grown under low and high irradiances [100 and 1 000 μmol(photon) m^{-2} s^{-1}]. Three measuring spectral regimes were applied using a *PAM 101* fluorometer: (*1*) excitation in the red region (maximum at the wavelength of 649 nm) and detection in the far-red region beyond 710 nm, (*2*) excitation in the blue region (maximum at the wavelength of 461 nm) and detection beyond 710 nm, and (*3*) excitation in the blue region and detection in the red region (660–710 nm). Non-photochemical quenching of maximal (NPQ) and minimal fluorescence (SV_0), determined by detecting Chl *a* fluorescence beyond 710 nm, were significantly higher for blue excitation as compared to red excitation. We suggest that this results from higher non-radiative dissipation of absorbed excitation energy within light-harvesting complexes of PS2 (LHC2) due to preferential excitation of LHC2 by blue radiation and from the lower contribution of PS1 emission to the detected fluorescence in the case of blue excitation. Detection of Chl *a* fluorescence originating preferentially from PS2 (*i.e.* in the range of 660–710 nm) led to pronounced increase of NPQ, SV_0, and the PS2 photochemical efficiencies (F_V/F_M and F_V'/F_M'), indicating considerable underestimation of these parameters using the standard set-up of *PAM 101*. Hence PS1 contribution to the minimal fluorescence level in the irradiance-adapted state may reach up to about 80 %.

Additional key words: *Hordeum*; non-photochemical quenching; non-radiative dissipation; *PAM* fluorometer; photosystems 1 and 2.

The pulse amplitude modulated technique for measurement of chlorophyll (Chl) *a* fluorescence at physiological temperatures is a widely used non-invasive method to monitor the functional state of photosynthetic organisms (Krause and Jahns 2003, Rosenqvist and van Kooten 2003). Nevertheless, there are some limitations that should be taken into account when interpreting the results of fluorescence analysis. Chl *a* fluorescence at room temperature reflects mainly emission from photosystem (PS) 2, but the contribution of fluorescence emanating from PS1 to the detected emission is not negligible when some commercially available fluorometers are used.

The room temperature emission spectrum of PS2 *in vivo* exhibits two bands with the maxima in the red and far-red region around 685 and 740 nm, while PS1 emission spectrum is characterized by one band with the maximum around 720 nm (Franck *et al.* 2002). Standard set-up of the pulse amplitude modulated fluorometer *PAM 101/103* (*H. Walz*, Effeltrich, Germany) involves emitter-detector unit detecting fluorescence in the far-red

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[***]Corresponding author; fax: +420 59 746 02 78, e-mail: Vladimir.Spunda@osu.cz
Abbreviations: Chl – chlorophyll; *clo f2 – chlorina f2* barley mutant; F_V/F_M and F_V'/F_M' – maximal photochemical efficiency of photosystem 2 for dark-adapted leaves and for leaves exposed to actinic radiation; HI – high irradiance; LED – light-emitting diode; LHC1 and LHC2 – light-harvesting chlorophyll *a/b*-binding protein complexes of photosystems 1 and 2; LI – low irradiance; NPQ – non-photochemical quenching of maximal fluorescence; NRD – non-radiative dissipation of absorbed excitation energy; PPFD – photosynthetically active photon flux density; PS1 and PS2 – photosystems 1 and 2; SV_0 – non-photochemical quenching of minimal fluorescence; WT – wild type.
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region beyond 710 nm. The main PS2 emission band is cut off and thus the contribution of PS1 emission to total fluorescence in the far-red region is not negligible. In this spectral region fluorescence originating from PS1 may represent up to 40 % signal in the state of minimal fluorescence (F_0) in C_3 plants and even 50 % signal in C_4 plants characterized by higher PS1/PS2 ratio than the C_3 species (Genty *et al.* 1990, Pfündel 1998, Agati *et al.* 2000, Peterson *et al.* 2001, Franck *et al.* 2002). In the state of maximal fluorescence (F_M) the maximal estimates of contribution of PS1 emission are about 10 % (Pfündel 1998, Agati *et al.* 2000, Franck *et al.* 2002).

As a consequence, the PS1 contribution affects the values of some fluorescence parameters. The formulas for coefficients of photochemical and non-photochemical quenching of Chl *a* fluorescence (q_P and q_N) directly imply the insensitivity of these parameters to PS1 emission. On the contrary, the maximal photochemical efficiency of PS2 in the dark- and irradiance-adapted state (F_V/F_M and F_V'/F_M'), actual efficiency of PS2 photochemistry (Φ_{PS2}), and non-photochemical quenching of F_M and F_0 based on Stern-Volmer formalism (NPQ and SV_0) are underestimated due to PS1 fluorescence (Genty *et al.* 1990, Pfündel 1998, Agati *et al.* 2000).

The objective of this study was to investigate the effects of PS1 emission contribution at different spectral region of excitation and detection of Chl *a* fluorescence on determination of the individual fluorescence parameters. In addition to standard set-up of *PAM 101/103* fluorometer we used emitter-detector unit *ED-101BL* employing a blue light-emitting diode (LED) as a source of excitation radiation and a filter slide that allows detection of fluorescence beyond 710 nm and also in the region without significant PS1 fluorescence, *i.e.* between 660 and 710 nm. A long-pass filter *RG9* (*Schott*, Mainz, Germany) was used for detection of fluorescence in the region beyond 710 nm and a long-pass filter *RG645* (*Schott*, Mainz, Germany) together with a short-pass heat absorbing filter *Calflex X* (*Balzers*, Liechtenstein) were used for monitoring fluorescence in the red region (660–710 nm). The standard set-up of *PAM 101/103* fluorometer involves emitter-detector unit *101-ED* with a red LED to excite fluorescence emission, which is passed through an *RG9* filter and thus detected beyond 710 nm. Emission spectra of the blue and red LED were recorded by a spectroradiometer *LI-1800* (*LI-COR*, Lincoln, NE, USA). The spectra were characterized by the maxima at the wavelengths of 461 and 649 nm with corresponding half peak bandwidths of 27 and 22 nm for blue and red LED, respectively (data not shown).

We chose plants with different sizes of light-harvesting complexes of PS2 (LHC2) and PS1 (LHC1), *i.e.* wild-type barley (*Hordeum vulgare* L. cv. Bonus; WT) and its Chl *b*-less mutant *chlorina f2* (*clo f2*). The consequence of the absence of Chl *b* in this mutant is the depletion of some proteins of both LHC2 and LHC1. The functional Chl antenna sizes of PS1 and PS2 in the mutant are reduced by 30 and 80 %, respectively (Harrison *et al.* 1993). In addition, the preferential reduction of LHC2 with respect to LHC1 was achieved by the cultivation of plants under high irradiance (HI) (Čajánek *et al.* 2002). The plants were grown from seeds under controlled environment conditions inside growth chamber (*HB 1014*, *Bioline-Heraeus*, Germany) at the two photosynthetic photon flux densities (PPFDs) [100 and 1 000 μmol(photon) m^{-2} s^{-1}], 20 °C, 65 % relative humidity, and 16/8 h day/night regime. The primary leaves of 7–11-d-old plants were used for all the measurements.

Room temperature Chl *a* fluorescence was measured using *PAM 101/103* fluorometer on the detached leaf segments placed inside the leaf-disc oxygen electrode chamber (*LD2/2*, *Hansatech Instruments*, King's Lynn, UK) with the adaxial side up. The following fluorescence parameters were calculated: the maximal photochemical efficiency of PS2 for dark-adapted leaves [$F_V/F_M = (F_M - F_0)/F_M$] and for leaves exposed to a given actinic radiation [$F_V'/F_M' = (F_M' - F_0')/F_M'$]; non-photochemical quenching of F_M ($NPQ = F_M/F_M' - 1$) (Bilger and Björkman 1990) and F_0 ($SV_0 = F_0/F_0' - 1$) (Gilmore and Yamamoto 1991). F_0 and F_M are the minimal and maximal fluorescence levels for the dark-adapted (at least 1 h) plants; F_0' and F_M' are the minimal and maximal fluorescence levels determined at steady state of fluorescence upon exposure to a given actinic radiation [100, 230, and 450 μmol(photon) m^{-2} s^{-1}]. For F_M and F_M' determinations, the saturating "white light" pulses of 0.8 s duration and incident PPFD of approximately 5 000 μmol(photon) m^{-2} s^{-1} were applied. Actinic and saturation radiation were provided by *KL 1500* halogen lamp (*Schott*, Mainz, Germany).

Acclimation responses of the photosynthetic apparatus of WT and *clo f2* plants to different growth irradiances were characterized in our previous studies (Kurasová *et al.* 2002, Štroch *et al.* 2004a). Here we focus on the effects of different spectral region of excitation and detection of Chl *a* fluorescence on the estimation of excitation energy utilization within PS2. While Φ_{PS2} did not significantly alter using different spectral regions of excitation as well as detection of Chl *a* fluorescence (data not shown), the other fluorescence parameters were affected by the measuring regime. For all plant variants, NPQ estimated from far-red detected Chl *a* fluorescence was significantly higher for blue excitation than for red excitation at all applied incident irradiances (by 13–42 %) (Fig. 1). SV_0 was the most sensitive parameter to the choice of spectral region of Chl *a* fluorescence excitation. For WT grown under low irradiance (LI) the effect of blue excitation resulted in slight increase of SV_0 (by 19–26 %) in comparison to red excitation. In the case of HI-grown WT, SV_0 for blue excitation increased relative to red excitation more notably than in LI-grown WT (by 47–59 %). For *clo f2* plants grown under both LI and HI about two-fold SV_0 values were found at the blue excitation.

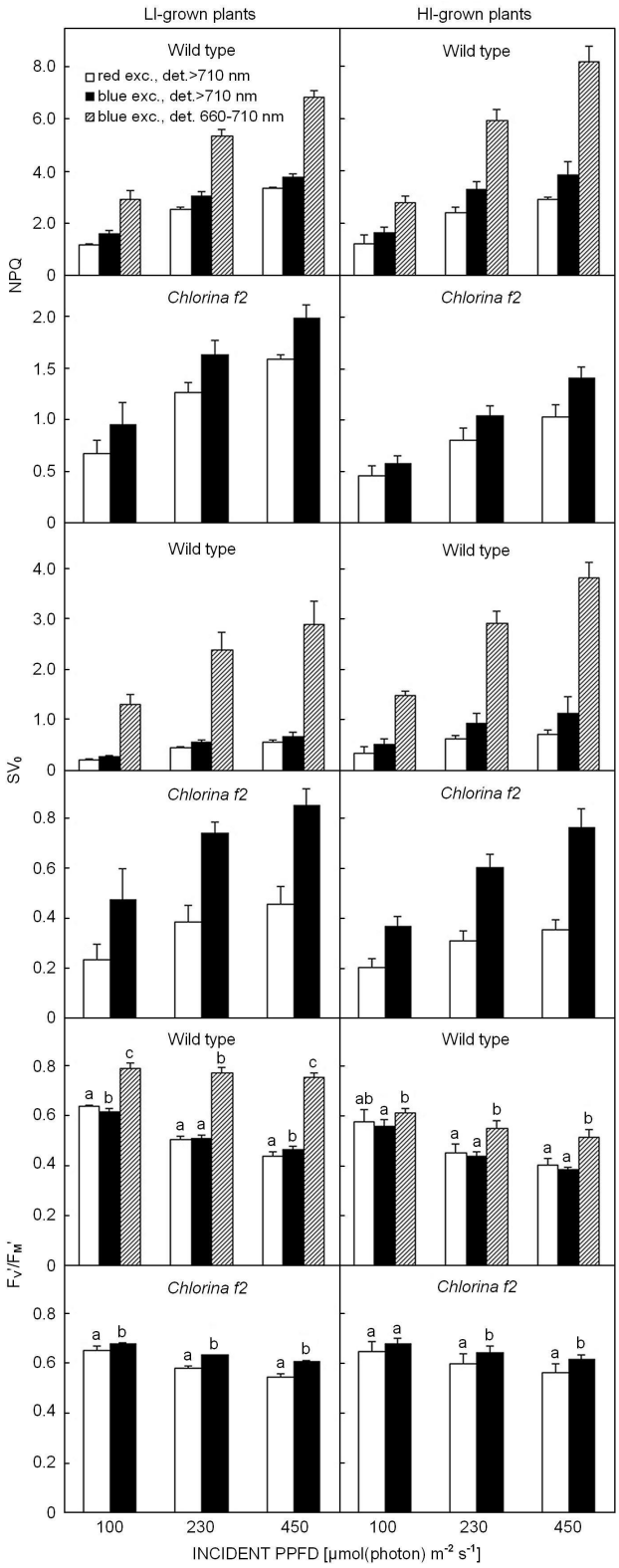

Fig. 1. Non-photochemical quenching of maximal fluorescence (NPQ) and minimal fluorescence (SV_0) and photochemical efficiency of open photosystem 2 reaction centres (F_V'/F_M') for barley wild-type and *chlorina f2* mutant grown under low irradiance [100 µmol(photon) m^{-2} s^{-1}; LI] and high irradiance [1 000 µmol(photon) m^{-2} s^{-1}; HI]. Fluorescence parameters were determined at steady state of chlorophyll *a* fluorescence that was recorded for different spectral regions of excitation and detection. Data followed by the same letter at the given incident PPFD indicate non-significant difference (p>0.05; Student's *t*-test). All differences between the data at the given incident PPFD for NPQ and SV_0 are significant at p<0.05 (Student's *t*-test). n = 3–5±SD.

LHC2 is the site of non-radiative dissipation of absorbed excitation energy (NRD) that is monitored as non-photochemical fluorescence quenching (Müller *et al.* 2001, Štroch *et al.* 2004b). Thus, NRD localized within LHC2 estimated from NPQ and particularly SV_0 was higher for blue excitation. From the emission spectra of LEDs we can deduce that radiation emitting by a blue LED of the *ED-101BL* unit excites mainly Chl *b* and carotenoids, whereas red radiation provided by a LED of the standard *101-ED* unit excites only Chls. We suppose that LHC2 is preferentially excited by blue radiation. In addition, the above mentioned changes in SV_0 demonstrated that the contribution of PS1 to the fluorescence emission excited by red radiation increased with extent of LHC2 reduction. Acclimation of WT to HI led to the characteristic increase of SV_0 that was more pronounced for blue excitation. At the highest incident irradiance [450 µmol(photon) m^{-2} s^{-1}] that induced the maximal SV_0 values the increase of SV_0 for HI plants in comparison with LI ones amounted 24 and 65 % for red and blue excitation, respectively. Accordingly, preferential excitation of LHC2 by blue radiation renders SV_0 more sensitive parameter to estimate changes of NRD within LHC2 upon acclimation to HI.

The lower contribution of PS1 emission for blue excitation as compared to red excitation may also explain higher F_V/F_M and F_V'/F_M' values for blue excitation (Figs. 1 and 2). However, this result was not observed in all cases. For example, in both WT and *clo f2* plants grown under HI, the difference of F_V/F_M for red and blue excitation was statistically non-significant. Using red and blue excitation of Chl *a* fluorescence we monitored different populations of chloroplasts within the leaf. Compared to blue radiation, red radiation penetrates deeper into the leaf and excites also chloroplasts in the lower cell layers (Buschmann and Lichtenthaler 1998, Vogelmann and Evans 2002). Fluorescence originating from chloroplasts deeper inside the leaf is captured as well as fluorescence from the top layers of chloroplasts because the detected emission (*i.e.* far-red region) is not re-absorbed. It is possible that the gradient of PS2 photoinhibition within the leaf was developed in plants grown under HI. If we take into consideration a greater degree of PS2 photoinhibition within chloroplasts in the upper cell layers than in the lower layers, then F_V/F_M for red excitation should be higher than that for blue excitation. This effect may be responsible for the suppression of the difference of F_V/F_M values that was found for LI-grown plants. Because of deeper penetration of red radiation into the leaf the effect of re-absorption of PS2 fluorescence by Chls within PS1 is enhanced for red excitation and may slightly contribute to the greater PS1 emission in the region above 710 nm (Agati *et al.* 2000).

When the red fluorescence emission (660–710 nm) was detected, the expected trend of changes of fluorescence parameters was observed. Unfortunately, fluorescence in this spectral window was not detectable for the *clo f2* mutant due to a low content of Chl *a* that is for LI-grown *clo f2* only about half as high as for WT (Štroch *et al.* 2004a). The contribution of PS1 fluorescence was minimal within 660–710 nm and therefore F_V/F_M and F_V'/F_M' reached higher values (Figs. 1 and 2). F_V/F_M for WT grown under LI was 0.887 that is significantly higher than values reported for plants in optimal physiological state using standard set-up of *PAM 101/103* fluorometer. Our result is in agreement with the F_V/F_M of PS2 that was calculated to be 0.88 (Pfündel 1998).

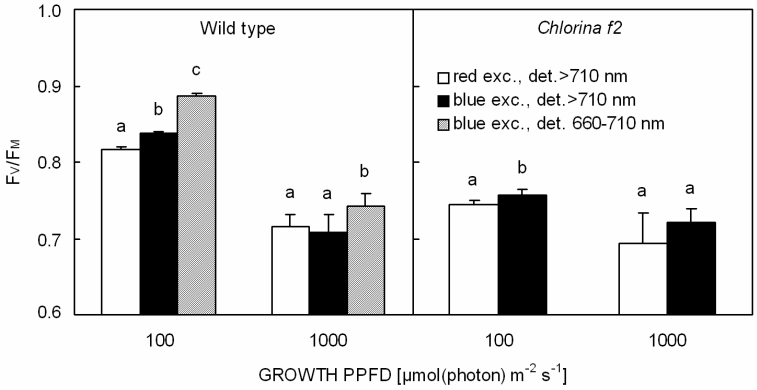

Fig. 2. The maximal photochemical efficiency of photosystem 2 (F_V/F_M) for dark-adapted barley wild-type and *chlorina f2* mutant grown under low irradiance [100 µmol(photon) m^{-2} s^{-1}] and high irradiance [1 000 µmol(photon) m^{-2} s^{-1}] determined for different spectral regions of excitation and detection of chlorophyll *a* fluorescence. Data followed by the same letter at the given growth PPFD indicate non-significant difference (p>0.05; Student's t-test). n = 4–11±SD.

Both NPQ and SV_0 exhibited surprisingly large increase, when only the red fluorescence was detected (Fig. 1). As expected, SV_0 was more sensitive to PS1 emission than NPQ, because PS1 emission contributes to the total fluorescence much more in the F_0 state than in the F_M state. Whereas NPQ values for the detection in the red region were higher by 70–113 % as compared to

those for the detection in the far-red region at the same excitation, SV_0 values exhibited 2.8–4.7 fold increase. We suppose that the extremely high values of NPQ are partly due to underestimation of F_M'. Under high NRD the closure of all PS2 reaction centres probably requires higher intensity of saturating pulse than was applied in our experiments. The obtained data revealed that the contribution of PS1 emission in the far-red region in the state of open PS2 reaction centres strongly increased after induction of NRD. Using SV_0 values determined for detection of Chl *a* fluorescence in the red and far-red region at the blue excitation we performed a model calculation in which negligible contribution of PS1 emission was considered within 660–710 nm. In addition, we assumed that the extent of PS1 fluorescence is invariable during transition from darkness to the state adapted to a given actinic radiation, when NRD is induced. Based on these presumptions, almost 70 and 80 % of the F_0' signal detected in the far-red region for plants grown under HI and LI, respectively, should be attributed to the PS1 emission. Thus, under the induction of irradiance-saturated NRD within LHC2 the contribution of PS1 fluorescence to the F_0' signal can strongly exceed the PS2 fluorescence. We found almost two-fold contribution of PS1 emission in the F_0' state than is that calculated from the data of Genty *et al.* (1990). This may be explained partially by the different spectral region of excitation of Chl *a* fluorescence, because Genty *et al.* (1990) used yellow LED filtered through a 580 nm band-pass filter (half peak bandwidth 40 nm) as a source of excitation radiation in contrast to blue LED that provides more direct excitation of the LHC pigments.

In conclusion, we showed that different spectral regions of both excitation and detection of Chl *a* fluorescence at room temperature significantly affected absolute values of some fluorescence parameters, especially NPQ and SV_0. Using the standard set-up of *PAM 101/103* fluorometer (*i.e.* excitation in the red spectral region and detection beyond 710 nm) the underestimation of SV_0 due to excitation region was much more pronounced for the plants with reduced size of LHC2. Thus, the method using blue excitation of detected Chl *a* fluorescence is more suitable for qualitative estimation of the effects of acclimation to HI and defects of pigment-protein complexes assembly on the efficiency of NRD localized within LHC2. The substantial reduction of PS1 contribution to the detected emission achieved by the detection of Chl *a* fluorescence in the region of 660–710 nm resulted in the SV_0 values that correspond to the real efficiency of NRD within LHC2. The maximal value of SV_0 found for HI-grown WT (about 4, Fig. 1) agrees with the maximal efficiency of NRD which may reach up to 80 % (Bassi and Caffarri 2000).

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