PHOTOSYNTHETICA 42 (2): 161-172, 2004

The gradient of exciting radiation within a sample affects the relative height of steps in the fast chlorophyll *a* fluorescence rise

P. SUŠILA*, D. LAZÁR, P. ILÍK, P. TOMEK, and J. NAUŠ

Laboratory of Biophysics, Faculty of Sciences, Palacký University, tř. Svobody 26, 771 46 Olomouc, Czech Republic

Abstract

Measurement of the chlorophyll (Chl) *a* fluorescence rise (FR) under higher exciting irradiance (EI), the O-J-I-P transient, or under lower irradiance, the O-I-P transient, is a routinely used method to access photosystem 2 function in thylakoid membranes of chloroplasts. Our measurements with a suspension of pea thylakoid membranes showed that the relative heights of the J and I steps in the FR depended not only on EI but also on the concentration and thickness of the sample. We explain this effect as a consequence of the gradient of EI within the sample. We tested this suggestion by theoretical simulations of the FR based on the model that was previously used for simulation of the FR considering in addition the gradient of EI within the sample. Our theoretical results correspond well with the experiments. The irradiance gradient effect may influence measured FR significantly and this fact should be taken into consideration in the interpretation of measured FRs.

Additional key words: exciting irradiance gradient; model; O-J-I-P transient; optical properties; *Pisum*.

Introduction

Measurements of chlorophyll (Chl) *a* fluorescence are often used to study the function of photosynthetic apparatus (for reviews see Krause and Weis 1991, Govindjee 1995). Among fluorescence methods, fluorescence induction is the most frequently used because it is non-invasive, sensitive, fast, easily measured, relatively cheap, and brings much information about the function of photosynthetic apparatus (for reviews see Govindjee 1995, Schreiber *et al.* 1995, Lazár 1999, Samson *et al.* 1999). Fluorescence induction consists of fluorescence rise (FR) from the so-called O level to the P level, which is completed within several seconds (in dependence on the used exciting irradiance, EI) and from the subsequent slow decrease (within several minutes) of fluorescence from the P level to the terminal steady-state level T.

When the mechanical-shutter fluorometers using a low EI or the shutter-less fluorometers with a high EI are used, the typical O-I-P (Forbush and Kok 1968, Munday and Govindjee 1969) or O-J-I-P (Strasser and Govindjee 1992a,b) transients, respectively, can be detected with unstressed plant material at room temperature. A dip denoted as D can be also sometimes observed after the first step in the curve under both low and high irradiances (Munday and Govindjee 1969, Schreiber and Neubauer 1987). The fluorescence rise from the O level to the first step measured under both low and high EI reflects predominantly the photon driven reduction of the primary quinone electron acceptor of photosystem 2 (PS2), Q_A (Forbush and Kok 1968, Melis 1985, Cao and Govindjee 1990, Hsu 1992, Lavergne and Leci 1993, Lazár *et al.* 1997, Stirbet *et al.* 1998, Tomek *et al.* 2001). However, this initial rise can also be influenced by the function of the donor side of PS2 (Hsu 1993, Lavergne and Leci 1993) and by energetic connectivity between PS2 units (Lavergne and Leci 1993). On the other hand, the subsequent fluorescence increase after the I step (under low irradiance) or after the J step (under high irradiance) up to the P level reflects an accumulation of reduced electron carriers beyond Q_A (Munday and Govindjee 1969, Neubauer and Schreiber 1987, Baake and Schlöder 1992,

Received 17 October 2003, *accepted* 10 March 2004.
*Corresponding author; fax: +420-585 225 737, e-mail: susila@prfnw.upol.cz
Abbreviations: Chl, chlorophyll; EI, exciting irradiance; F(t), fluorescence intensity at time t; F_{2ms}, fluorescence intensity at 2 ms, which is the typical time of the J step appearance; F_{30ms}, fluorescence intensity at 30 ms, which is the typical time of the I step appearance; F_0, minimal fluorescence intensity; F_P, maximal measured fluorescence intensity (fluorescence intensity at peak P); FR, fluorescence rise; PAR, photosynthetically active radiation; PS2, photosystem 2; V(t), relative variable fluorescence [$= (F(t) - F_0)/(F_P - F_0)$]; V_{2ms}, relative fluorescence at time 2 ms [$= (F_{2ms} - F_0)/(F_P - F_0)$]; V_{30ms}, relative fluorescence at time 30 ms [$= (F_{30ms} - F_0)/(F_P - F_0)$].
Acknowledgements: This work was supported by grants MSM 153100010 and FRVS 1569 from the Ministry of Education of the Czech Republic. D.L. thanks Grant Agency of Czech Republic for financial support (grant no. 204/02/P071).

Lazár *et al.* 1997, Stirbet *et al.* 1998, Tomek *et al.* 2001, Lazár 2003) and can reflect also other processes (Schreiber *et al.* 1989, Schreiber and Krieger 1996, Koblížek *et al.* 2001, Strasser *et al.* 2001, Pospíšil and Dau 2002, Bukhov *et al.* 2003, Heredia and De Las Rivas 2003, Schansker *et al.* 2003, Vredenberg and Bulychev 2003). Consideration of some other processes in understanding of the FR also implied from theoretical simulations of the FR based on so far most complex model of reactions occurring in PS2 (Lazár 2003) because only the reactions occurring in PS2 could not result in simulation of well resolved O-J-I-P transient as routinely measured. The transformation of the O-I-P transient obtained under low irradiance to the O-J-I-P transient measured under high irradiance was studied in detail by Tomek *et al.* (2001).

Generally, measurement of any fluorescence signal can be affected by optical effects in the measured sample, such as photon absorption, dispersion, reflectance, transmission, and focusing (for a review of the optical effects in a leaf see Vogelmann 1993). Due to these effects, an irradiance gradient is formed within a sample. This gradient has been modelled theoretically (Seyfried and Fukshansky 1983, Terashima and Saeki 1985, Hirota 1987a,b, Fukshansky *et al.* 1992) and measured experimentally (Vogelmann and Björn 1984, Vogelmann *et al.* 1988, 1989, Cui *et al.* 1991). It was shown that irradiances in the most important spectral regions of the exciting radiation, *i.e.* the blue and red ones, decrease exponentially within the leaf tissue (*e.g.* Bornman *et al.* 1991, Karabourniotis *et al.* 1999). This conclusion is supported also by application of the Kubelka-Munk theory of radiation spreading in diffuse media (Latimer and Noh 1987, Fukshansky *et al.* 1991). However, the pattern depends on the character of incident radiation (collimated or diffuse), on the presence of palisade parenchyma (Vogelmann and Martin 1993), or on the focusing action of epidermal cells (for review see Vogelmann 1993).

Several papers also presented measurements of the Chl fluorescence profile within a leaf. A profile of steady state fluorescence level has been obtained using a fibre optic probe (*e.g.* Bornman *et al.* 1991) and the influence of Chl fluorescence on the gradient of penetrating radiation in a leaf has been evaluated (Lork and Fukshansky 1985). A detailed measurement of Chl fluorescence spectral profile using confocal microscopy was conducted by Pfündel and Neubohn (1999) and by microscopic images of leaf cross sections by Takahashi *et al.* (1994) and Koizumi *et al.* (1998). The steady state fluorescence intensity or spectral characteristics were detected in the above mentioned cases.

Another question appears regarding the influence of the EI gradient on measured FR. So far, the effect of the EI gradient on the FR measured in presence of DCMU was theoretically described (Malkin *et al.* 1981, Dvořák *et al.* 1992). Recently, Hsu and Leu (2003) suggested on the basis of experimental measurements that the J-I phase of the O-J-I-P FR is caused by the EI gradient within the sample. Hence, the goal of this work was to examine whether the EI gradient can affect the measured FR [O-(J)-I-P curve]. We have found that the shape of the FR curve depends on Chl content and thickness of used suspension of thylakoid membranes and we suggest that this is caused by the gradient of EI within the sample. We tested this suggestion by means of theoretical simulations. Our theoretical results correspond with the experiments.

Materials and methods

Preparation of thylakoid membranes: Pea seedlings (*Pisum sativum* L.) were cultivated hydroponically at 20 °C in perlite substrate supplied with water at a light regime of 16 h "white light" [90 μmol(photon) m^{-2} s^{-1} of PAR] measured with Quantum Radiometer *LI-189* (*LI-COR*, Lincoln, U.S.A.)/8 h dark. The leaves were harvested after 14 d of growing.

Thylakoid membranes were prepared using the modified procedure of Dau *et al.* (1995). Fresh detached pea leaves (22 g) were homogenised using a *T25* homogeniser (*IKA Labortechnik*, Staufen, Germany) three times for 5 s at 367 rps. The homogenization medium contained 0.4 M sucrose, 0.4 M NaCl, 35 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 1 mM EDTA, 5 mM ascorbic acid, and bovine serum albumin (2 kg m^{-3}). The homogenate was filtered through 4 layers of miracloth (42 μm mesh diameter) and centrifuged in the cooled centrifuge at 5 000×*g* for 6 min. The resulting pellet was resuspended in the medium containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 8 mM $MgCl_2$, and centrifuged (5 000×*g*, 10 min). Thylakoid membranes were finally resuspended in a small amount of the R-medium [50 mM MES (pH 6.0), 15 mM NaCl, 10 mM $MgCl_2$]. Thylakoid membranes were stored in a flask placed in ice water. The whole procedure was performed under dim green radiation at 4 °C.

The Chl (*a*+*b*) concentration in the suspension of thylakoid membranes was determined spectrophotometrically in 80 % acetone with a spectrophotometer *Lambda 40* (*Perkin-Elmer*, Norwalk, USA) according to Lichtenthaler (1987). The Chl concentration in the stock suspension was 480.000 g m^{-3}. The thylakoid membranes were gradually diluted to reach Chl concentrations of 240.000, 120.000, 60.000, 30.000, 15.000, 7.500 3.750, 1.875, and 0.938 g m^{-3}.

Measurements of fluorescence rise: FR curves were measured with the suspension of thylakoid membranes at room temperature using the shutter-less Chl fluorometer PEA with the PEA/VA vial adapter for measuring on liquid-phase samples (*Hansatech Instruments*, Norfolk, England). 6 bright "red light" emitting diodes (LED;

emission maximum at about 650 nm) arranged in a circle below the measuring cuvette excited fluorescence. These exciting beams are focused on the bottom of the measuring cuvette in the PEA measuring head. The diameter of the irradiated sample area was 12.2 mm. The maximal EI was about 12 000 μmol(photon) m^{-2} s^{-1} of PAR (measured with Quantum Radiometer *LI-189*, *LI-COR*, Lincoln, USA) at the bottom of the measuring cuvette. Fluorescence was detected by the PIN photodiode in the direction perpendicular to the bottom of the measuring cuvette (the angle of the exciting beams and detected fluorescence was about 33°).

Different contents of Chl in thylakoid membrane suspensions (see section above) and irradiances of 100, 50, 25, and 12 % of the maximum provided by the fluorometer were used for the measurements of FR curves. Further, volumes of the suspension of 0.25, 0.50, and 1.00 cm^3 were used to modify the thickness of the sample in the centre of cylindrical cuvette (PEA accessory, 12.2 mm internal diameter, slightly concave bottom) to be 1.6, 3.8, and 8.1 mm, respectively. Thylakoid membranes were dark-adapted for 15 min before the measurement of FRs. For every measurement, always a new suspension prepared from the stock suspension was used.

The fluorescence signal was distorted by PEA fluorometer due to existence of non-zero offset signal as was checked with acetone Chl extracts of different Chl concentrations and sample thicknesses. However, this offset signal was for given sample constant starting from 50 μs to the end of the measurement. From this reason, the experimental FR curves are presented starting with 50 μs and the fluorescence signal at this time was supposed to be the minimal fluorescence F_0. Further, the existence of constant offset signal for a given sample (which can be different for different Chl concentrations, EI, and sample thicknesses) had, however, no effect on the course of the relative variable fluorescence V(t) [$= (F(t) - F_0)/(F_P - F_0)$] used for presentation of our experimental data and on evaluated V_{2ms} and V_{30ms} parameters. It is because the offset signal is additive to all elements in the expression for V(t) and hence the offset is cancelled out.

Measured FR curves with variable fluorescence lower than 100 PEA output units were excluded from further evaluation of the V_{2ms} and V_{30ms} parameters due to elimination of error of the analogue-digital conversion. This condition permits maximal error of the V_{2ms} and V_{30ms} parameters evaluation caused by the conversion to be about 1 %, when compared to full relative variable fluorescence range.

Results

The FRs measured with suspension of thylakoid membranes under different irradiances and Chl concentrations are shown in Fig. 1. FRs are expressed by means of the relative variable fluorescence $V(t) = [F(t) - F_0)/(F_P - F_0)]$. Symbols F(t), F_0, and F_P are the measured fluorescence intensity at time t, the minimal fluorescence intensity, and the maximal fluorescence intensity, respectively. When high irradiance is used for the measurements, the FRs show the typical O-J-I-P transient (Fig. 1*A*), which is changed into the O-I-P transient when measured under low irradiance (Fig. 1*D*). This gradual change of the shape of the FR caused by changes in EI is in agreement with previous results (see, *e.g.*, Strasser *et al.* 1995, Tomek *et al.* 2001). Under high EI, the F_P value reached the F_M value where all PS2 centres were closed.

Fig. 1 also shows that the relative heights of the J and I steps decrease with increasing amount of thylakoid membranes (expressed by means of Chl content). The results were measured with the sample volume of 0.5 cm^3, which corresponds, in our measurement geometry, with the sample thickness of 3.8 mm (see Materials and methods). A qualitatively similar effect was also obtained for other sample thicknesses (1.6 and 8.1 mm; values not shown).

The measured FRs were characterized by the parameters V_{2ms} and V_{30ms} defined as $(F_{2ms} - F_0)/(F_P - F_0)$ and $(F_{30ms} - F_0)/(F_P - F_0)$, respectively. $F_{2ms(30ms)}$ is the fluorescence intensity at 2 ms (30 ms), which is the typical time of the J (I) step in the FR under high EI.

The experimental dependences of the V_{2ms} and V_{30ms} parameters on the amount of thylakoid membranes, sample thickness, and incident EI are presented in Fig. 2. Several general trends can be distinguished in the courses of both V_{2ms} and V_{30ms} parameters. The first is a decrease of V_{2ms} and V_{30ms} values with decreasing EI (Fig. 2*A–D* or *E–H*). The second, dominant effect is a decrease of these parameters with increasing Chl concentration in the samples of constant thickness. The third effect is a decrease of these parameters with increasing sample thickness (see triangles *versus* squares for given sample concentration in Fig. 2). Moreover, it seems that the decrease of the parameters, caused either by the increasing sample amount or sample thickness, reaches saturation, which is characteristic for the given irradiance.

To explain the changes in the courses of measured FRs (Fig. 1) that are also demonstrated by the changes of V_{2ms} and V_{30ms} parameters (Fig. 2) we can take into account the optical properties of the sample. As the PEA fluorometer detects fluorescence at wavelengths above 700 nm, and because photon absorption by the photosynthetic apparatus of green plants is very low at these wavelengths, the changes in the FRs cannot be caused by re-absorption of fluorescence. We suggest that the changes in the FRs are caused mainly by the effect of the gradient of EI within the sample.

The sample can be divided theoretically into imaginary layers. The first layer partly absorbs the incident photons and lowers the irradiance incident on the second

layer, and so forth. Each layer of the sample thus acts as an inner filter for the EI generating a decreasing profile of EI within the sample. Finally, the FR curve detected by the fluorometer is in fact a sum of the FR curves originating from different layers of the sample. The quantitative course of the irradiance profile within a sample depends on the incident irradiance, Chl content, and also on the thickness of the sample. However, note that the photon gradient defined strictly by mathematical definition as a derivative d*I*/dx (x is the distance along the radiation beam, *I* is the irradiance) depends neither on the incident irradiance nor on the thickness of the sample.

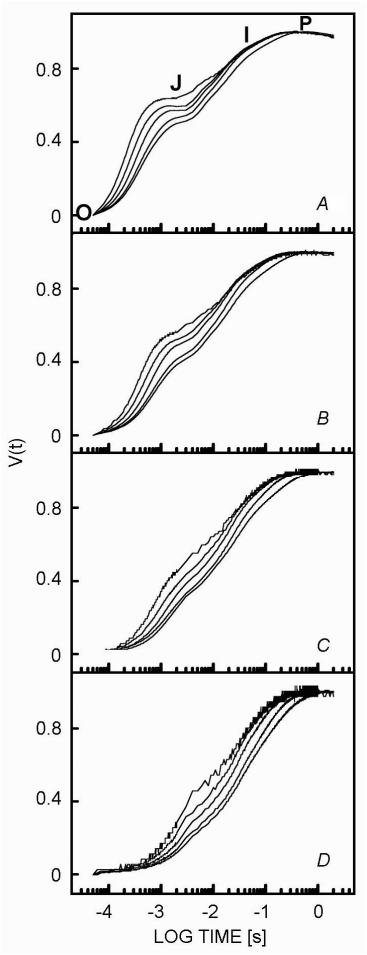

Fig. 1. Experimental fluorescence rise curves presented by means of the relative variable fluorescence V(t) measured with a layer of thylakoid membranes suspension of thickness 3.8 mm upon 100 (*A*), 50 (*B*), 25 (*C*), and 12 (*D*) % exciting irradiance. The contents of chlorophylls in the suspension were 7.5, 30.0, 60.0, 120.0, and 480.0 g m^{-3} (*from top to bottom*). The O, J, I, and P steps are denoted.

To test the hypothesis on the decisive role of the EI gradient, we have performed theoretical simulations of FRs considering the effect of the gradient of EI within the sample. For theoretical simulations of the FR we have used a model developed by ourselves (Lazár *et al.* 1997), which already successfully simulated changes in the O-J-I-P curve due to herbicide action and due to a different incident EI (Tomek *et al*. 2001). However, in principle any other model that successfully simulates the O-J-I-P curve can be used for these simulations. A scheme of our model, its solution, and rationale used for evaluation of the EI gradient within the sample are described in the Appendix.

Fig. 3 shows the theoretical FRs [presented by means of the relative variable fluorescence, V(t)] simulated for different irradiance and Chl contents and considering the EI gradient within the sample. Also presented are the FR curves simulated without regard to the EI gradient. A gradual change of the O-J-I-P transient simulated for high EI (Fig. 3*A*) to the O-I-P transient upon low irradiance (Fig. 3*D*) can be simulated by this model and the simulated FR curves qualitatively correspond well with the experimental FR curves (Fig. 1). Fig. 3 also shows that the gradient of EI in the model causes an attenuation of particular steps in the FR (lower four curves in each panel) when compared to the FR curve simulated without regard to the gradient of EI (the upper curve in each panel). This effect results from a contribution of fluorescence signals from deeper layers, characterized by lower relative heights of the J and I steps in FR, to the overall FR.

In accordance with the experiments, the relative height of the J and I steps in simulated FRs increases with decreasing Chl concentration in samples of the same thickness. Similar conclusions could be made from simulations for sample thicknesses of 1.6 mm and 8.1 mm (data not shown).

To quantify the effect of the gradient of EI within the sample on the simulated FR, we plotted the V_{2ms} and V_{30ms} parameters in dependence on Chl content, sample thickness, and irradiance in Fig. 4. Both the V_{2ms} and V_{30ms} parameters decreased with increasing Chl content for all considered sample thicknesses and irradiance. The V_{2ms} and V_{30ms} parameters reached maximal values for lower Chl contents. These maximal values correspond to the values obtained for the FR without consideration of the gradient of EI within the sample (see the *crosses* in Fig. 4). On the other hand, the saturation in decrease of the V_{2ms} and V_{30ms} parameters appeared for higher Chl contents. For higher sample thicknesses the saturation appeared at lower Chl contents. It means that at both limiting Chl contents the FR curves approach a limit shape determined by the irradiance only. Thus, the FR curve acquires a shape between the two extreme shapes—the shape for the case without regard to the gradient of EI and the shape for the case considering the gradient at saturation.

The courses of the V_{2ms} and V_{30ms} parameters calculated from the simulated FRs considering the gradient of EI within the sample (Fig. 4) correspond with the courses of the parameters obtained from the measured FRs with the suspension of thylakoid membranes (Fig. 2), especially for lower EI (panels *C*, *D*, *G*, and *H* of Figs. 2 and 4).

The only exception to this correspondence are the experimental concentration dependences of the V_{2ms} and V_{30ms} parameters calculated from FRs for lower Chl contents, high irradiances, and lower sample thicknesses (Fig. 2*E,F*). Instead of the expected monotonous decrease of these values with increasing Chl content there is

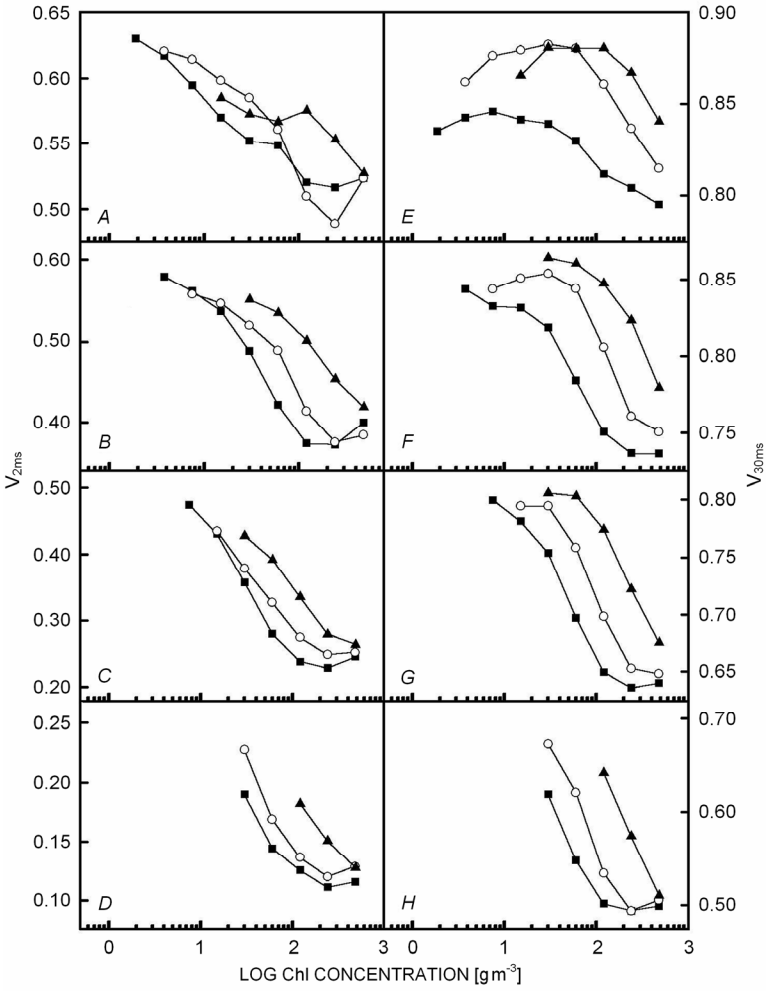

Fig. 2. Relative values of fluorescence signal at 2 and 30 ms presented by means of the V_{2ms} (*A–D*) and V_{30ms} (*E–H*) parameters, respectively, as calculated from the experimental fluorescence rise (FR) curves measured upon 100 (*A, E*), 50 (*B, F*), 25 (*C, G*), and 12 (*D, H*) % of incident exciting irradiance in dependence on the concentration of chlorophylls in thylakoid membranes' suspension. *Squares*, *circles*, and *triangles* denote the sample layer thickness of 8.1, 3.8, and 1.6 mm, respectively. The parameters were evaluated only from FR curves with variable fluorescence higher than 100 units (see Materials and methods).

a slight initial increase. In our opinion, this phenomenon is caused mainly by the experimental conditions that differ slightly from the theoretical ones. The exciting beam is not collimated but convergent. The rays in the convergent exciting beam can be for example partly reflected by the water-air interface when leaving the suspension. The reflected radiation can excite other parts of the sample than the primary beam and the resulting fluorescence adds to that primarily excited fluorescence. This additional fluorescence is characterized by lower values of V_{2ms} and V_{30ms}. Increasing sample concentration causes a higher scatter of EI inside the volume that lowers the effects of convergence and reflection from the water-air interface. It leads to more homogeneous radiation and the experimental dependences behave in accordance with the theoretical predictions. Similarly, in thicker samples, the effect of the interface is much less important, because the irradiance at this interface is lower.

Discussion

Our experimental results show that the course of the FR depends not only on irradiance used for the measurements (see, *e.g.*, Tomek *et al.* 2001) but also on the content and thickness of the measured suspension of thylakoid membranes. The experimental dependences of the V_{2ms} and V_{30ms} parameters on the Chl content and sample thickness can be simulated satisfactorily by assuming a gradient of EI along a cylindrical measured sample.

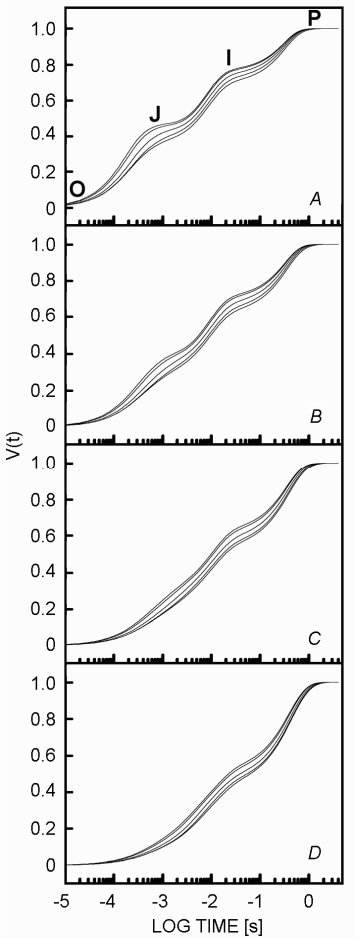

Fig. 3. Theoretical fluorescence rise (FR) curves presented by means of the relative variable fluorescence V(t) simulated for 100 (*A*), 50 (*B*), 25 (*C*), and 12 (*D*) % of incident exciting irradiance (EI) assuming gradient of EI within sample thickness of 3.8 mm (*lower four curves*). The assumed contents of chlorophylls were 480.0, 60.0, 30.0, and 7.5 g m^{-3} (*from bottom to top*). *The upper curves* in each panel are simulations of FRs, not assuming the gradient of EI. The O, J, I, and P steps are denoted.

The theoretical simulations used here for the interpretation of the FRs with thylakoid membrane suspensions can be of importance also for the FR measurements with leaves. In leaves, however, many other effects influence and contribute to the gradient of EI. Among these effects the heterogeneity in distribution of PS2s within the leaf, radiation scattering, and inner reflections (Nauš *et al.* 1989, 1993), surface reflections (McClendon and Fukshansky 1990a), the sieve effect (McClendon and Fukshansky 1990b), and the effective optical path length (Agati *et al.* 1993, Richter and Fukshansky 1994) should be named. Whereas the Chl content in liquid samples usually used for FR measurements, *i.e.* in algae suspensions, is about 10–15 g(Chl) m^{-3} (*e.g.* Strasser *et al.* 1995), the Chl content in an average green leaf is about 300 mg(Chl) m^{-2} (*e.g.* Ilík *et al.* 2000), which corresponds to 3 kg(Chl) m^{-3}, assuming 0.1 mm thickness of the leaf.

We used our homogeneous theoretical model also for the simulation of the FR of an average green leaf having the properties mentioned above. We simulated the FRs for 12–100 % EI and calculated the V_{2ms} and V_{30ms} values. V_{2ms} and V_{30ms} declined by about 30–15 and 15–8 %, respectively, compared to magnitudes calculated from FRs simulated without regard to the gradient of EI (not shown). On the other hand, for the alga or thylakoid suspension [with 15 g(Chl) m^{-3}], that can be described well by our homogeneous model, the EI gradient decreases V_{2ms} and V_{30ms} by about 21–8 % and 10–4 %, respectively, when compared to the values evaluated without the gradient (for a thickness of 8.1 mm; see Fig. 4).

The relative values V_{2ms} and V_{30ms} only characterize the FR curves and are not usually used for further calculations. However, the values of the fluorescence signal at 100 and 300 μs are used for detailed analysis of energy conversion in the photosynthetic apparatus in the JIP test (Strasser and Strasser 1995), which is used to address the question of the biotic and abiotic stresses of plant material (*e.g.* Appenroth *et al.* 2001, Nussbaum *et al.* 2001). We evaluated the values of $V_{100\mu s}$ and $V_{300\mu s}$ [$= (F_{100\mu s(300\mu s)} - F_0)/(F_P - F_0)$, where $F_{100\mu s(300\mu s)}$ is fluorescence intensity at 100 (300) μs] from the FR curves simulated for the alga or thylakoid suspension [15 g(Chl) m^{-3}] and for the leaf [3 kg(Chl) m^{-3}] for 100 % EI considering and without the gradient of EI. For the alga suspension the $V_{100\mu s}$ and $V_{300\mu s}$ values declined by about 26 and 21 % (calculated for the thickness of 8.1 mm), respectively, and by about 37 and 32 %, respectively, for the leaf when compared to the values of the parameters evaluated from the FR simulated without a consideration of the gradient. Our results show that the gradient of EI might also significantly affect the results of the JIP test. Hence, in case of a stress action that affects the content of Chls (and thus the gradient), conclusions made on the basis of a comparison of results of the JIP test obtained with unstressed and stressed samples should be taken with care.

A further parameter that is evaluated from the FR is the fluorescence level at the I-step measured at low EI. At this condition the I-step reflects predominantly the photochemical closure of the Q_B-non-reducing PS2 centres

(centres that cannot reduce further electron acceptors beyond Q_A) (Forbush and Kok 1968, Melis 1985, Tomek *et al.* 2001) and thus the height of the I-step relative to the maximal fluorescence (F_M) determines the relative number of the Q_B-non-reducing PS2 centres (Melis 1985, Cao and Govindjee 1990). We already stressed the effect of EI without consideration of the photon gradient within the sample on determination of the relative number of the Q_B-non-reducing PS2 centres (Tomek *et al.* 2003). To extend the description of the effect of EI on the determination of the relative number of the Q_B-non-reducing PS2 centres, we simulated FR for thylakoid or alga suspension [15 g(Chl) m^{-3}] with a sample thickness of 8.1 mm and for the average green leaf [3 kg(Chl) m^{-3}, thickness of 0.1 mm] under 3 % irradiance considering the EI gradient within the sample. The relative height of the I-step was evaluated as relative height of fluorescence signal at 30 ms, *i.e.* the V_{30ms} parameter. The V_{30ms} parameter declined by about 11 % for the suspension and by about 20 % for the leaf compared to the values calculated from the FRs simulated without regard to the EI gradient. These results again show that the gradient of EI can also significantly affect the determination of the relative amount of the Q_B-non-reducing PS2 centres. Hence, the same as in the case of the JIP test mentioned above, when a stress action changes the content of Chls, the calculated amount of the Q_B-non-reducing PS2 centres should be interpreted with care.

Recently Hsu and Leu (2003) suggested on the basis of experimental measurements that the EI gradient within the sample could be the origin of the J-I phase in the O-J-I-P FR. Even if our both experimental and theoretical results clearly show that the EI gradient within the sample can significantly affect the O-J-I-P FR, our theoretical simulations did not prove the suggestion made by Hsu and Leu. Moreover, recent results show that rather other processes than the EI gradient are responsible for the I step in the FR (Schreiber *et al.* 1989, Strasser *et al.* 2001, Pospíšil and Dau 2002, Bukhov *et al.* 2003, Heredia and De Las Rivas 2003, Schansker *et al.* 2003,

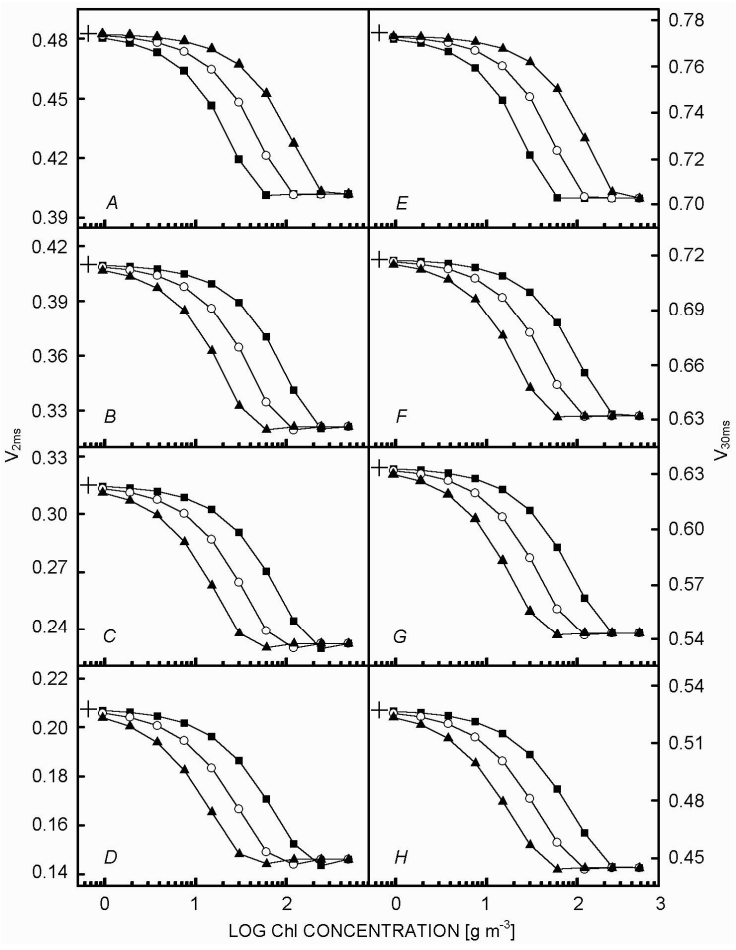

Fig. 4. Relative values of fluorescence signal at 2 and 30 ms presented by means of the V_{2ms} (*A–D*) and V_{30ms} (*E–H*) parameters, respectively, as calculated from the theoretical fluorescence rise (FR) curves simulated for 100 (*A, E*), 50 (*B, F*), 25 (*C, G*), and 12 (*D, H*) % of incident exciting irradiance (EI), assuming a gradient of EI within sample thickness of 8.1 mm (*squares*), 3.8 mm (*circles*), and 1.6 mm (*triangles*). *Crosses in the top left corner of each panel* indicate values of the parameters evaluated from the FR curves simulated without assumption of the gradient of EI.

Vredenberg and Bulychev 2003).

All our experimental and theoretical results are suited for the case of low contribution of radiation scattering, reflections, and other optical effects (sieve effect, heterogeneity of concentration, *etc*.). These effects have been partly neglected in the presented model. Another project would be desirable to evaluate the possible contribution of the mentioned additional optical effects on the FR.

In conclusion, our results show that the gradient of EI can significantly affect the course of FR and thereby also the values of the parameters determined from FR for suspension of functional thylakoid membranes. The presented model may have some implications for the measurements with alga suspensions and leaves, where the irradiance gradients are also present. Thus, any conclusion made on the basis of parameters calculated from the FR should take into account the possible influence of photon gradient within the sample. Our results also imply that fitting of experimental FRs, measured mainly for the samples with a high Chl content (such as leaves), by theoretical models could be unsuccessful due to the omission of the gradient of EI within the sample.

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Appendix

Description of theoretical model: The model originally introduced by Lazár *et al.* (1997) but with slight modifications due to simulations of the effect of irradiance on the FR, as described in Tomek *et al.* (2001), was used for simulations of FRs in this work. A scheme of the model is shown in Fig. A1. Fluorescence yield at time t in the model can be expressed as follows (for detailed explanations of the notation of particular quinone forms see the legend to Fig. A1):

$$Y'_{I_k}(t) = \frac{(1-p)\, e'_{light}(t)}{1-p\, e'_{light}(t)} + e'_{dark}(t) + \left[Q_A^- I\right], \quad (1)$$

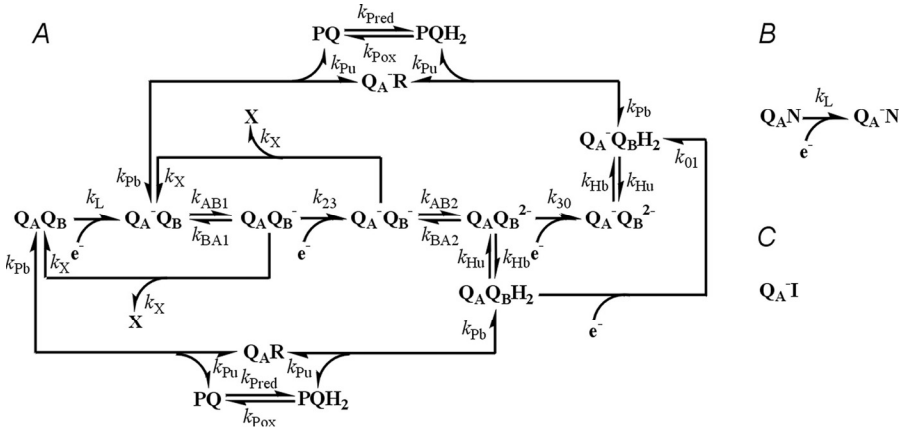

Fig. A1. Scheme of the model (adapted from Lazár *et al.* 1997) used for simulations of the fluorescence rise (FR) curves for the Q_B-reducing (part *A*), the Q_B-non-reducing (part *B*), and the inactive PS2 centres (part *C*; it is assumed that these PS2s are initially closed). $Q_A^{(-)}Q_B^{(-,\,2-)}$ means different redox forms of primary and secondary quinone acceptors while $Q_A^{(-)}Q_BH_2$ means protonated forms. Q_AR means centres without bound Q_B of the Q_B-reducing PS2 centres and Q_AN means the Q_B-non-reducing PS2 centres, while Q_A^-I means the inactive (in our terminology) PS2 centres. PQ and PQH_2 denote oxidised and reduced plastoquinone molecules, respectively. X expresses (unknown) component (Stirbet and Strasser 1996) that accepts electrons from single reduced Q_B with the rate constant k_X. The meaning of the other rate constants is as follows: k_L is irradiance dependent rate constant for Q_A reduction when Q_B is oxidised (in the Q_B-reducing PS2 centres) or not present (the Q_B-non-reducing PS2 centres); k_{23}, k_{30}, and k_{01} are the irradiance independent rate constants for subsequent Q_A reductions related to $S_2 \rightarrow S_3$, $S_3 \rightarrow S_0$, and $S_0 \rightarrow S_1$ transitions of oxygen evolving complex, respectively (assumption of the model; see Lazár *et al.* 1997); k_{AB1}, k_{AB2} are the rate constants of forward electron transport from Q_A^- to Q_B and Q_B^-, respectively; k_{BA1}, k_{BA2} are the backward rate constants of the previous reactions; k_{Hb}, k_{Hu} are the rate constants of binding and unbinding, respectively, of $2H^+$ to/from Q_B^{2-}; k_{Pb}, k_{Pu} are the rate constants of binding and unbinding, respectively, of plastoquinone molecule to/from the Q_B-pocket of PS2; k_{Pox}, k_{Pred} are the rate constants of the plastoquinone pool oxidation and reduction, respectively. The initial amounts of starting forms were normalised to 1 ($[Q_AQ_B]_0 = 0.66$, $[Q_AN]_0 = 0.22$, and $[Q_A^-I]_0 = 0.12$; this corresponds to 75 % of the Q_B-reducing and 25 % of the Q_B-non-reducing of all PS2 centres that can reduce Q_A (88 % of all PS2 centres), and 12 % of the inactive PS2 centres, respectively, and the initial amount of PQ form was 7 (it corresponds to 7 plastoquinone molecules per one PS2). The initial amounts of all other forms were equal to zero. In accordance to Lazár *et al.* (1997), the following values were used in the simulations: $k_X = 5\,000\ s^{-1}$, $k_{23} = 1\,250\ s^{-1}$, $k_{30} = 500\ s^{-1}$, $k_{01} = 20\,000\ s^{-1}$, $k_{AB1} = 3\,500\ s^{-1}$, $k_{AB2} = 1\,750\ s^{-1}$, $k_{BA1} = 175\ s^{-1}$, $k_{BA2} = 35\ s^{-1}$, $k_{Hb} = 150\ s^{-1}$, $k_{Hu} = 100\ s^{-1}$, $k_{Pb} = 100\ s^{-1}$, $k_{Pu} = 150\ s^{-1}$, $k_{Pox} = 1\ s^{-1}$, $k_{Pred} = 1\ s^{-1}$.

where

$$e'_{light}(t) = \left[Q_A^- Q_B\right](t) + \left[Q_A^- N\right](t)$$

and

$$e'_{dark}(t) = \left[Q_A^- Q_B^-\right](t) + \left[Q_A^- Q_B^{2-}\right](t) + \left[Q_A^- Q_B H_2\right](t) + \left[Q_A^- R\right](t)$$

and the subscript I_k indicates the exciting irradiance (EI) as described by Eq. (3) in the section below that is proportional to the value of rate constant k_L of irradiance dependent Q_A reduction. For more detailed description of the model and its assumptions see Lazár *et a*l. (1997) and Tomek *et al.* (2001).

Calculation of gradients of EI within sample and the final FR: We assumed that the only effect that affects FRs measured at different conditions as shown in Fig. 1 is a gradient of EI within the measured sample. Further assumption is that PS2s are homogeneously distributed within the whole volume of measured sample, a condition that is fulfilled in thylakoid membrane suspension. Assuming the Lambert-Beer attenuation law and neglecting reflection on sample surface, the irradiance within the sample at distance d [cm] perpendicular to the irradiated surface of the sample can be expressed as

$$I(d) = I_0\, 10^{-\gamma c d}, \tag{2}$$

where I_0 is incident irradiance on the surface of the sample, γ is a constant determined by absorption of radiation by the sample [$m^3\ g^{-1}\ cm^{-1}$], and c is the content of absorbing particles [$g\ m^{-3}$] in the sample. From the absorption measurement of the thylakoid membranes suspension of different contents of Chls at 650 nm, we evaluated the mean value of the γ to be 0.035 $m^3\ g^{-1}\ cm^{-1}$, which we have used for all further calculations. The quantity γ includes both the absorption and the scattering attenuation of the directly spreading radiation. However, the contribution of the scattered radiation to the excitation of fluorescence was not included in our model.

The contents of Chls used in the calculations were the same as in the experiments, *i.e.* c = 0.938, 1.875, 3.750, 7.500, 15.000, 30.000, 60.000, 120.000, 240.000, and 480.000 g m^{-3}. For the simulation of the FR of the average green leaf (see Discussion) we used the Chl content of 3 kg m^{-3} and the sample thickness of 0.1 mm. We assumed that the fluorescence signal from the part of the sample that is irradiated by $I_0/100$ and smaller is negligible. Thus, for the given values of γ and c we calculated from Eq. (2) the distance $d_{critical}$, which fulfils this constrain. If overall sample thickness $D < d_{critical}$ then the whole sample was considered and when $d_{critical} < D$, only the thickness of the sample equal to $d_{critical}$ was considered in further calculations.

When the sample of thickness D (or $d_{critical}$) is divided into n layers with the same thickness denoted as l (= D/n or = $d_{critical}/n$) then the irradiance at the surface of the layer k (k = {1, 2, .., n}) can be expressed as

$$I_k = I_0\, 10^{-\gamma c l (k-1)}. \tag{3}$$

Irradiance calculated according to Eq. (3) also determines the value of rate constant k_L that is used for simulation of the FR originating from particular layer. The k_L value used for 100 % irradiance was 4 000 s^{-1} (Tomek *et al.* 2001) and was proportionally decreased when the irradiance was decreased. A particular FR curve was simulated for each layer using the model described in the section above.

Further, we assumed that the fluorescence signal from the layer k can be expressed as

$$F_k(t) = \left(I_k - I_{k+1}\right) Y_{I_k}(t), \tag{4}$$

where I_k, I_{k+1} are calculated according to Eq. (3) and units of irradiance can be arbitrary. As we did not assume the effect of fluorescence re-absorption (see Results), the final simulated FRs coming from all considered layers of the sample as they are presented in Fig. 3 are sums of FRs coming from these layers and can be expressed as

$$F_{final}(t) = \sum_{k=1}^{n} F_k(t), \tag{5}$$

where $F_k(t)$ is calculated according to Eq. (4).

For determination of the minimal number of layers which should be considered to obtain correct simulation of the $F_{final}(t)$ we simulated $F_{final}(t)$ for the given values of I_0, γ, c, and d assuming gradually 2–40 layers (step of 2) of the sample and calculated corresponding V_{2ms} and V_{30ms} parameters. We found that while the V_{2ms} and V_{30ms} parameters did not change much for lower Chl contents, they decreased with the number of layers for Chl contents above about 100 g m^{-3}. We found that the values of the V_{2ms} and V_{30ms} parameters calculated for number of layers higher than 10 differ by no more than 1 %. Therefore, we divided the considered part of the sample (see above) to 10 layers for all simulations of the $F_{final}(t)$.

In summary, the entering parameters of the model were those listed in the legend to Fig. A1. The connectivity parameter p was 0.55, the same as used in Lazár *et al.* (1997). The value of the rate constant k_L was varied to simulate

the different incident irradiance for the particular layer. The FR coming from the particular layer was calculated for the given k_L according to Eq. (4) and summary (final) FR coming from all sample layers was calculated according to Eq. (5). All theoretical simulations were done using a routine written in Visual Basic for Microsoft Excel. The Euler method with constant time step of 10 μs was used for the integration of differential equations describing the theoretical model.