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On the advantages of using green light to study fluorescence yield changes in leaves

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Abstract

In photosynthetic chains, the kinetics of fluorescence yield depends on the photochemical rates at the level of both Photosystem I and II and thus on the absorption cross section of the photosynthetic units as well as on the coupling between light harvesting complexes and photosynthetic traps. A new set-up is described which, at variance with the commonly used set-ups, uses of a weakly absorbed light source (light-emitting diodes with maximum output at 520 nm) to excite the photosynthetic electron chain and probe the resulting fluorescence yield changes and their time course. This approach optimizes the homogeneity of the exciting light throughout the leaf and we show that this homogeneity narrows the distribution of the photochemical rates. Although the exciting light is weakly absorbed, the possibility to tune the intensity of the light emitting diodes allows one to reach photochemical rates ranging from 10^4 s^{-1} to 0.25 s^{-1} rendering experimentally accessible different functional regimes. The variations of the fluorescence yield induced by the photosynthetic activity are qualitatively and quantitatively discussed. When illuminating dark-adapted leaves by a weak light, the kinetics of fluorescence changes displays a pronounced plateau which precedes the fluorescence increase reflecting the full reduction of the plastoquinone pool. We ascribe this plateau to the time delay needed to reduce the photosystem I electron acceptors.

Keywords: Photosynthetic chain; Fluorescence; Photochemical quenching; Non-photochemical quenching; Electron transfer

The measure of the light-induced changes of fluorescence yield is a widely resorted approach to study the photosynthetic processes *in vivo* (see [1–4] for reviews). These changes are mostly controlled by the redox states of the photosystem II electron acceptor or electron donor. Consequently, their kinetics under continuous illumination provide information on the various fluxes involved in the function of the photosynthetic apparatus such as the excitation transfer between the light harvesting complexes and the reaction centers, the electron flux along the photosynthetic chain, the electrochemical transmembrane potential resulting from the metabolic activities (either photosynthetic or respiratory) and the Benson and Calvin cycle involved in the fixation of CO_2 (see e.g. [5]). The large variety of parameters controlling the changes of fluorescence yield makes this observable extraordinary powerful but, also, dramatically hinders a rigorous and accurate interpretation. The aim of this paper is to describe a new set-up designed to measure light-induced fluorescence yield changes *in vivo* and to discuss its practical merits. We will illustrate some of the various parameters controlling these changes and attempt to disentangle their respective contributions.

Since the first fluorescence kinetic measure performed by Kaustky and Hirsch [6] a large variety of technique has been developed to characterize the photosynthetic fluorescence yield changes. Describing or discussing the various methods that have been developed is beyond the scope of the present paper, rather we will focus on the basic principles which underlie the measure of the light-induced changes of fluorescence yield. In order to detect fluorescence emission, the sample has to be illuminated. Since photosynthesis utilizes light as a substrate to

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; F_0, fluorescence yield in the dark; F_{max}, maximum fluorescence yield; LED, light emitting diodes; PS I and PS II, Photosystem I and II, respectively; Φ_{PSII}, Photosystem II quantum yield; Q_A, primary quinone acceptor in Photosystem II; Y_Z, tyrosine D_1-160 of Photosystem II

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drive electron transfer reactions this illumination may induce as well electron transfer reactions in the photosynthetic chain. This mere statement has led to two different strategies for the design of fluorimeters. Most commonly, the continuous illumination sources provide both the exciting light that drives the photosynthetic process and the detecting light that induces fluorescence. In this case the wavelength of the continuous light has to be significantly shorter than the fluorescence wavelength (i.e. shorter than 660–900 nm) to permit its filtering out. Alternatively, two separate light sources may be used to independently induce the photosynthetic process and probe the fluorescence yield. This allows one to measure not only the light-induced fluorescence yield changes but also the recovery of the initial fluorescence yield after an illumination. Here again the measure of the fluorescence emission requires these two light sources to be filtered out. This may be achieved either by using an appropriate combination of filters or by measuring the modulated fluorescence emission induced by a modulated light beam of sufficiently low intensity to induce a negligible electron flux (see [7] and references therein). This latter technical approach has however the strong drawback of limiting the time resolution of the technique to the frequency of the modulated source. The time resolution issue is important to take as much benefit as possible of the richness of the observable. Indeed, illuminating the photosynthetic chain induces various fluxes and the resulting fluorescence changes are expected to depend on the rate of the limiting step in the chain. This is because the redox state of the Photosystem II cofactors, and thus of the fluorescence induction curve, is a function of the ratio between their reduction rate (i.e. the actinic light intensity) and their oxidation rate that is controlled by the rate of the limiting-step. As a consequence, varying the light intensity and thus the photochemical rate allows one to probe different steps among the entire electron transfer chain, provided the time resolution of the set-up is suited. As will be described, with the present set-up the intensity of the light source that is used to induce the photosynthetic process covers several orders of magnitude and its time resolution has been be tuned down to 4 μs without decreasing the signal to noise ratio.

1. Materials and methods

1.1. Biological material

The time course of fluorescence changes can be analyzed with entire leaf either cut or attached to the plant. The leaf is placed at the interface between two chambers in which a gas with a controlled concentration of oxygen, nitrogen and CO_2 can be continuously flowed. When studying fluorescence properties of liquid samples such as unicellular algae or isolated thylakoids, a 2-mm-thick cuvette can be used and placed in the sample holder.

1.2. Optical device

The leaf is illuminated by an array of 15 green light emitting diodes (LEDs HLMP-CM15) (peak emission at 530 nm, band width at half maximum 50 nm) mounted in a conical device. The emitted light is thereby focused onto a circular aperture (diameter 5 mm) on which the leaf is applied. Alternatively, laser flashes may be used as an exciting light (650 nm, half duration 8 ns). These are provided by a dye laser pumped by the second harmonic of a Nd:Yag laser, coupled to a light pipe that can be placed in the center of the LED array. The LED emission in the red part of the spectrum is filtered out by a filter Schott BG39, 3 mm thick. Fluorescence light is detected from the face opposite to the illuminated one by a photodiode Hamamatsu S3590-08. The actinic light transmitted through the leaf is filtered out by the combination of a Long Pass reflecting filter (50% transmission at 650 nm) and a Schott filter RG665, 3 mm. This combination decreases the light intensity at the level of the RG665 Schott filter which would otherwise itself fluoresce and thus contribute significantly to the overall fluorescence. The contribution of the florescence emission induced by the excitation of the Schott filter is thereby negligible as compared to fluorescence emitted by chlorophyll so that the F_0 level may be determined with good accuracy.

The intensity of the electrical current supplying the LED's can be tuned between 15 μA and 260 mA , which respectively translate into light intensities ranging from 2 to 15000 $\mu E/m^2/s$. The LED power supply is computer-controlled and allows light intensity changes in less than 1 μs.

1.3. Detection of the fluorescence signal

The output signal of the photodiode is amplified with relative gains of 1, 14, 45, and 4400 with time constants of 3.4 μs, 9 μs, 45 μs and 400 μs, respectively. The output signal is sampled by an analog digital-converter (18 bits). If needed, the signal to noise ratio can be improved by digital integration i.e. by summing n samples ($1 < n < 100$) separated by time intervals, the duration, t, of which can be set to match the analog time constant. The time resolution of the method Δt is equal to the product $n \times t$. By adjusting the analog time constant, the value of n and t, the time resolution of the method can be discretely tuned from ~4 μs ($n=1$) (for the highest light energy) to 40 ms (for the lowest light energy). Since the fluorescence intensity is sampled at discrete times after the onset of the exciting light, the time courses may be studied over a time range extending from the ms to several tens of minutes. The actinic light intensity can also be tuned from ~15 000 to ~0.3 $\mu E/m^2/s$ (10^4 to 0.2 photon/s per PS II centers) with a signal to noise ratio better than 10^3.

The fluorescence intensity depends both on the intrinsic fluorescence yield of the sample and on the exciting light intensity. When large currents are used (>3 mA, ~400 $\mu E/m^2/s$), the light intensity output of the LEDs decreases slightly as a function of the illumination time. For a given illumination sequence, the instrument function $I=f(t)$ is determined by monitoring the fluorescence intensity of a fluorescing dye sample with a time-independent fluorescence

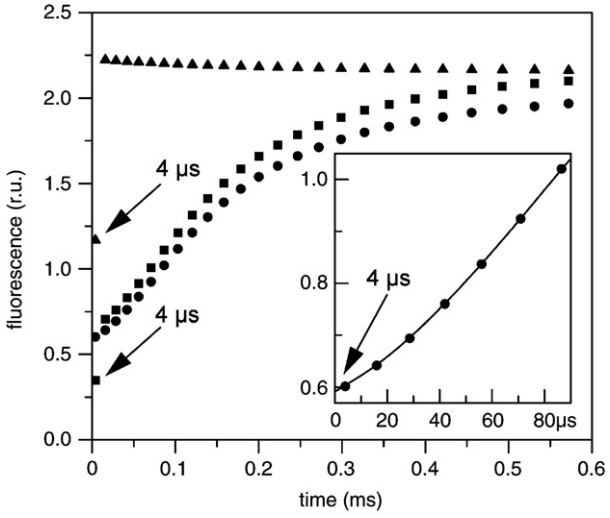

Fig. 1. Correction of the fluorescence changes by the instrument function. The instrument function (triangles) is obtained by submitting a fluorescent dye to the same illumination as the sample. Dividing the fluorescence changes measured with the sample (squares) by the instrumental function corrects for the variation in light intensity as shown with the circles. The inset shows a zoom on the early fluorescence changes after correction and illustrates the time resolution of the set-up.

yield. The fluorescence changes induced by illumination of the biological sample are then divided by this instrument function $I=f(t)$ in order to correct for possible variation in the exciting light intensity. This allows one to determine the changes in the fluorescence yield as a function of the illumination. Yet, the possible variation in photochemical rates resulting from the changes in the light intensity are neglected. Fig. 1 gives an example of such a correction. A dark-adapted leaf from *Arabidopsis thaliana* was submitted to a strong illumination. Fig. 1 displays the initial part of the fluorescence induction kinetics obtained before (squares) or after division (circles) by the instrumental function $I=f(t)$ (triangles). The insert shows the first data points and illustrates the noise of the set-up that is below 10^{-3} for a single sweep. We note that taking into account the instrument function which is partly determined by the analogic time constant, improves the time resolution of the set-up which can be shorter than the analogic time constant of the amplifier (5 μs in this experiment) (compare squares and circles).

The possibility to trigger short exciting light pulses (10 μs duration) also allows sampling the fluorescence yield in the dark. The intensity of the detecting flashes is tuned to hit less than 1% of PS II centers so that the amount of photo-induced charge separation is kept negligible thereby allowing the estimation of the intrinsic fluorescence yield.

2. Results and discussion

2.1. On the advantages of a green source as an excitation light

One of the key parameters which controls the kinetics of fluorescence changes is the photochemical rate constant of photosystem II (k_{PSII}). This rate is linearly related to the probability of exciton trapping by a PS II. This probability obviously depends on the local intensity of illumination and on the antenna size. Because the light intensity decreases with light absorption, this intensity is expected to be more even throughout the leaf when illuminating the leaf with a weakly-absorbed excitation source like green light (peak emission at 530 nm) than with blue or red light which is strongly absorbed by chlorophyll. Fig. 2 shows the fluorescence changes induced by the illumination with a green light of the adaxial face of a dark-adapted leaf.

The illumination intensity was such that the rate of the photosynthetic process under steady state condition was limited by the photochemical reactions. It has been known for long that, in dark-adapted leaves, the Benson and Calvin cycle is essentially inactivated. Consistent with this inactivation, a few seconds of illumination with a light of limiting intensity results in a large increase of the fluorescence yield indicating the progressive reduction of the plastoquinone pool. In other terms, the consumption of the reducing equivalent by the Benson and Calvin cycle is slower than their production by the photosynthetic chain. In curve 1, only the red-most part of the fluorescence emission was detected ($\lambda>750$ nm) in order to select the less efficiently reabsorbed fluorescence wavelengths. Under these conditions, all the chloroplasts should contribute about equally to the overall fluorescence intensity irrespective of their location within the leaf. Conversely, in curve 2, the fluorescence was detected through an interference filter, $\lambda_{max}=680$ nm, a wavelength close to the peak of the absorption by chlorophyll. Under these conditions, we expect fluorescence to mainly arise from those chloroplasts localized closed to the output (adaxial) face. The similarity of the fluorescence time course when detected in the far red (Fig. 2 curve 1) or at 680 nm

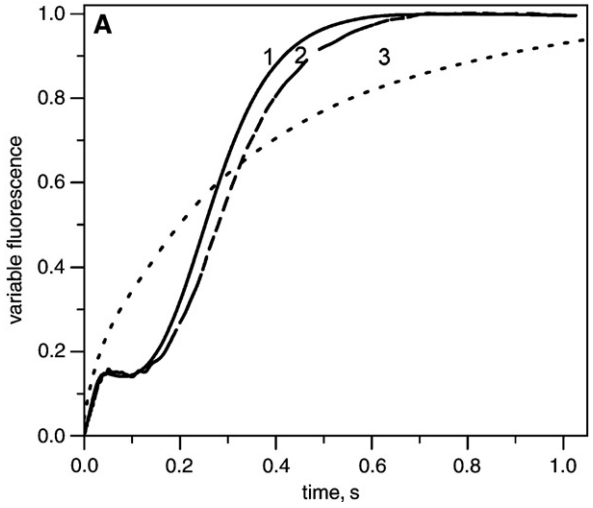

Fig. 2. Kinetics of fluorescence increase from an *Arabidopsis thaliana* leaf under moderate light excitation. The light intensity corresponded to $ki_{PSII}=\sim 65\ s^{-1}$. Before each experiment, the leaf was dark-adapted for more than 10 min. Curve 1: the leaf was illuminated by the adaxial (upper) face. The short wavelength range of the fluorescence emission spectrum was cut off by a RG 750 filter ($\lambda>750$ nm) so that only the red-most part of the fluorescence emission spectrum was detected. Curve 2: the fluorescence was detected at 680 nm (Interference filter λ_{max} 680 nm, FWHM 10 nm). Curve 3: The leaf was illuminated at 470 nm. Fluorescence light was detected from the illuminated face through a wide-band red filter (set up II, see text).

(Fig. 2 curve 2) suggests that the PS II photochemical rate hardly depends on the location of the illuminated chloroplasts within the leaf.

To further assess the kinetic distortion resulting from a heterogeneous illumination within the leaf we considered the extreme case of a strongly-absorbed actinic light. The dependence of the fluorescence time-course on the wavelength of the actinic excitation was characterized by comparing with the same leaf, the fluorescence changes with the set-up using green light as an exciting source and another set-up in which the exciting source is provided by blue LEDs (λ_{max} ~470 nm) and the fluorescence is detected from the illuminated face through a wide-band red filter. In both cases the leaf was illuminated by its adaxial face, the blue light intensity was tuned to reach similar k_{PSII} than with green light excitation. As illustrated by the comparison of curves 1 and 3, Fig. 2, the kinetics were significantly dependent on the excitation wavelength. Whereas a pronounced plateau could be resolved when using the green LEDs, it was blurred when using the blue ones. This may be rationalized if, in the latter case, the (various) thylakoids, which contribute to the overall fluorescence emission, are illuminated with various light intensities owing to the strong absorption of the exciting light. The progressive light absorption by the successive cell layers leads to a decreasing light intensity which is expected to be all the more pronounced as the exciting wavelength is strongly absorbed. The resulting overall fluorescence time course is then expected to be a combination of the time courses that would be induced by various light intensities. This illustrates to which extent a strongly absorbed actinic light results in a significant distortion of the fluorescence time

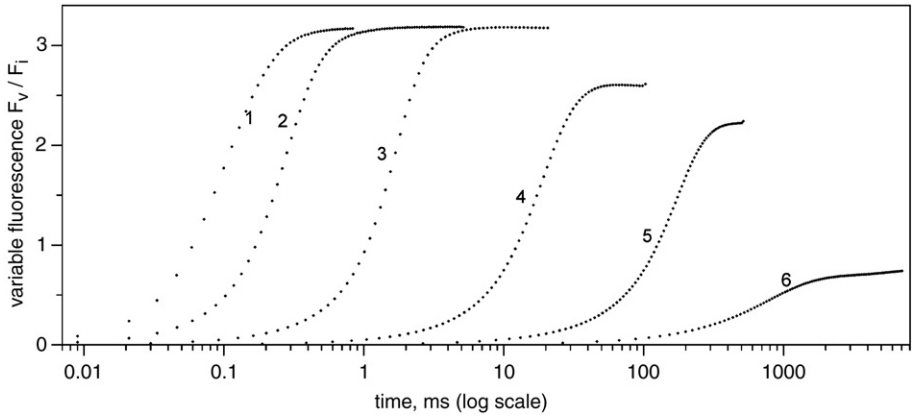

Fig. 3. Fluorescence increase measured from a young spinach leaf in presence of DCMU (40 μM) as function of the light intensity on a logarithmic time scale. Curve 1: $ki_{PSII} = \sim 10000\ s^{-1}$, corresponding approximately to 16 000 μE m^{-2} s^{-1}. Curve 2: $ki_{PSII} = \sim 3700\ s^{-1}$. Curve 3: $ki_{PSII} = \sim 550\ s^{-1}$. Curve 4: $ki_{PSII} = \sim 50\ s^{-1}$. Curve 5: $ki_{PSII} = \sim 5\ s^{-1}$. Curve 6: $ki_{PSII} = \sim 0.5\ s^{-1}$.

course owing to the progressive decrease of the actinic light intensity, and hence of the photochemical rate k_{PSII}, as it penetrates deeper into the leaf. Such distortion, which is obviously prejudicial to the analysis of the fluorescence changes since it blurs the kinetic parameters which govern them, may be avoided by using a weakly absorbed light, such as green light. Yet, it could be argued that green light has limited experimental merits since, being weakly absorbed, it constrains the range of photochemical rates that can be investigated. As we will show now, the recent outcome of powerful LEDs makes this reserve obsolete.

2.2. Fluorescence changes kinetics in the presence of DCMU under various light intensities

We have analyzed the fluorescence changes in the presence of a saturating concentration of DCMU within a large range of green light intensity (Fig. 3).

In the presence of DCMU, the overall fluorescence yield is mostly controlled by the redox state of the primary quinone acceptor of PS II (Q_A) [8]. Thus, the rate of fluorescence yield change is commonly considered as proportional to the exciton trapping efficiency of the PS II reaction centers and to the light intensity. If the latter is constant during the illumination, the relative trapping efficiency of PS II may be inferred from the dependence of the fluorescence yield on the product between the duration of the illumination and the light intensity ($I \times t$). In the log(t) scale, the fluorescence time course, measured with different light intensity, should thus be shifted one from the other by log(I) but should display the same shape irrespective of the light intensity. As shown in Fig. 3 with the lower intensities, the maximum fluorescence level decreased. This reflects a competition between the photochemical reduction of Q_A and its reoxidation through charge recombination processes. A closer look at curves 1 to 6 shows that only the initial part of the fluorescence induction curves is limited by the light-trapping process. Indeed, with the highest intensity (curve 1) we observed a significant slowing down in the tail of the time course compared to curve 2 or 3. This is illustrated in more details in Fig. 4 in which the fluorescence changes corresponding to curves 1–3 have been plotted on a linear scale as function of the $I \times t$ product.

As previously reported, the fluorescence rise observed in the presence of DCMU is not exponential as would be expected for the progressive closing of the PS II centers, but displays a marked sigmoidicity. As initially discussed in [9], this witnesses that, owing to the significant probability for exciton to hop from one closed photosystem II unit to an open one, the effective excitonic flux at the level of the opened photosystem II units increases as the number of the closed one increases. Interestingly, under the highest illuminations (Fig. 4, curves 1–2), the tail of the fluorescence induction kinetics is not proportional to $I \times t$, indicating that, at the highest intensity, a non-photochemical process, which occurs in the 100 μs time range, limits the rate of the fluorescence yield changes. This dark process is reminiscent of the slow fluorescence rise ($t_{1/2}$ ~30–80 μs) measured after a saturating flash given to thylakoids in the presence of DCMU [10–12]. This observation suggests the existence of a transient quenching state that is likely to be a

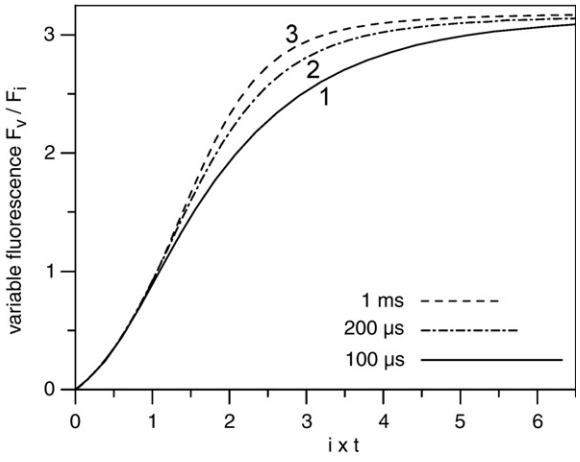

Fig. 4. Fluorescence increase measured from a young spinach leaf in presence of DCMU (40 μM) as function of the light intensity on a linear scale. Curves 1–3 same as curves 1–3 in Fig. 3.

particular redox state of PS II. As a candidate, we would like to discuss the likely role of a significant steady state concentration of P^{+}_{680} that would be formed during strong illumination. P^{+}_{680} has been known for long to be an as efficient quencher as the oxidized state of the primary quinone acceptor Q_A [13]. A slow fluorescence rise in the hundreds of μs time range has been observed in the presence or absence of DCMU [10–12], suggesting that a quencher, which was proposed to be P^{+}_{680} [11,12], decays in this time range. Consistent with this hypothesis, several authors reported that a significant fraction of P^{+}_{680} is effectively reduced in the μs to tens of μs time range owing to a small equilibrium constant between the $P^{+}_{680}Y_Z$ and $P_{680}Y^{ox}_Z$ states [14–18]. Alternatively, as a non exclusive hypothesis, the transient quenching state could be the $P_{680}Q_A$ state formed in the 100 μs time range by charge recombination in a fraction of PS II centers that inefficiently stabilize the charge-separated state [19].

As shown and discussed above, fluorescence time courses are strongly dependent on the light intensity. Consequently, knowing the precise value of the light intensity is essential when discussing such kinetics. The most commonly used unit for light intensity is μmole of photon s^{-1} m^{-2}. Yet, such unit has little experimental value since it cannot reliably be translated into a photochemical rate without knowing the absorbance of the sample, which is rarely the case. Obviously, similar light intensities expressed in photon s^{-1} m^{-2} are not expected to yield similar fluorescence time course if the exciting light is green, red or, even worse, white. There is thus, in our opinion, a real need for a more relevant unit which should be the number of electron transferred per unit of time and per PS II reaction center. As discussed now, the light intensity can be readily expressed in this unit. Indeed, theoretical simulations of fluorescence induction curves in the presence of DCMU have led to the phenomenological finding that the absorption of one photon per PS II induces a fluorescence increase of about $0.6(F_{max}-F_0)$ [9]. Interestingly, this hardly depends on the overall shape of the induction curve, or, in other words, on the connectivity between photosynthetic units. As an example, in the case of Fig. 4 curve 3, 1.8 ms of illumination are required to reach a fluorescence level of 0.6 $(F_{max}-F_0)$ corresponding to ~550 photons per PS II per second. Thus, under these conditions, the photochemical rates of PS II for Fig. 3 curves 1–6 are ~10 000 s^{-1}, ~3700 s^{-1}, ~550 s^{-1}, 50 s^{-1}, ~5 s^{-1}, ~0.5 s^{-1}, respectively. On this basis, the time constant of the non-photochemical process that kinetically limits the late fluorescence changes may be estimated to ~50–100 μs. Thus the large range of intensities that can be reached with bright LED's compensates the low absorption of the excitation wavelength and makes accessible, with a single detecting device, a wide range of photochemical rates.

2.3. The fluorescence time course as a tool to probe cytological parameters

We have argued that using a weakly absorbed light as an actinic source improves the homogeneity of the light intensity throughout the sample and thus narrows the distribution in photochemical rates. Yet, it is of note that significantly different fluorescence time-courses are obtained when illuminating the abaxial rather than the adaxial face of the leaf (Fig. 5 compare curves 1 and 2).

In this former case, the plateau was less pronounced and the tail of the induction curve was 2–3 fold slower. These two

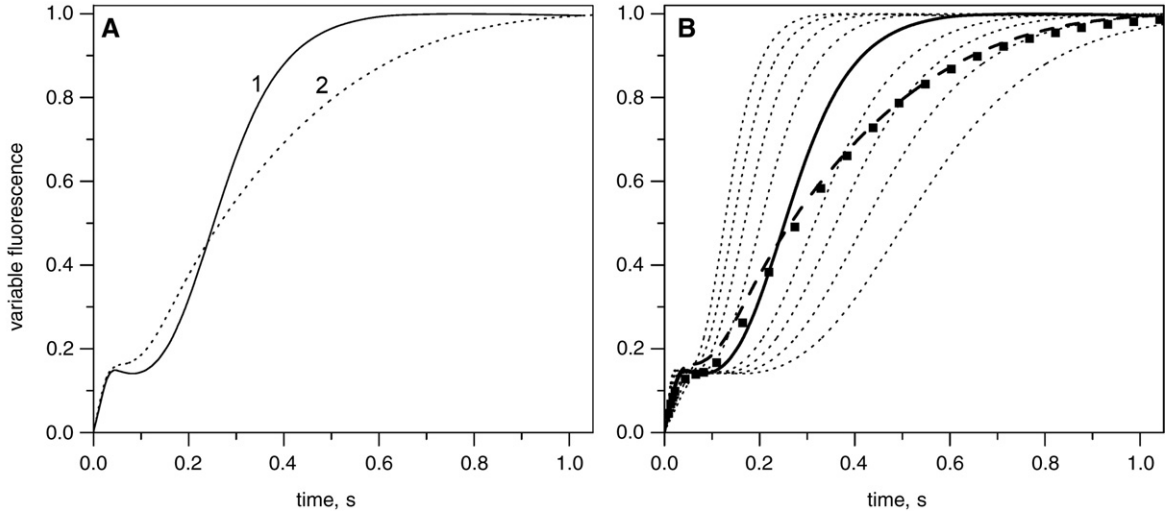

Fig. 5. Kinetics of fluorescence increase from an *Arabidopsis thaliana* leaf under moderate light excitation. The light intensity corresponds to ki_{PSII} = ~ 65 s^{-1}. Before each experiment, the leaf was dark adapted for more than 10 min. (A) Curve 1: the leaf was illuminated by the adaxial (upper) face. The short wavelength range of the fluorescence emission spectrum was cut off by a RG 750 filter (λ > 750 nm) so that only the red-most part of the fluorescence emission spectrum was detected. Curve 2: the leaf was illuminated by the abaxial face and fluorescence was detected at λ > 750 nm. (B) A simulation of the consequences of a heterogeneous illumination. When the light trapping rate is kinetically limiting the overall electron flux, a change in light intensity is formally equivalent to a homothetic transformation of the *x*-axis. The dotted lines were obtained as follows: the fluorescence time course corresponding to curve 1 was plotted against time after having multiplied the *x*-axis by a value ranging from 2 to 0.5 (the light intensity corresponding to that of curve 1 was arbitrarily set to 1). A linear combination of these various curves yields a fluorescence time course (squares) mimicking the experimental curve 2.

features may reflect a significant variation of k_{PSII} along the normal to the leaf plane. Indeed, the overall time course corresponding to Fig. 5A curve 2 may be simulated assuming that, for a given light intensity, the kinetics of fluorescence changes follows a time course similar to Fig. 5A curve 1 and that, at variance with our observation when the leaf is illuminated via the adaxial face, k_{PSII} varies to a significant extent from the entrance to the output face of the leaf. As shown in Fig. 5B, the linear combination of fluorescence time courses similar to that shown in Fig. 5A curve 1 induced by k_{PSII} varying from 0.5 to 2 (rel.un.) results in an overall kinetics similar to Fig. 5A curve 2.

A possible way to account for this finding of different fluorescence change time course when illuminating the abaxial or adaxial face, relies on the idea that light scattering depends on the light pathway throughout the leaf. As a support to this proposal, the cytological characteristics of the leaf tissue of the abaxial or adaxial faces are extremely different. The adaxial tissue is composed of palisade cells, oriented along the normal to the leaf plane. At variance with this well-ordered tissue, the abaxial tissue is composed of spongy mesophyll cells and large cavities. Whereas the palisade cells may act as light guides, this spongy mesophyll is likely to induce a strong scattering. We propose that, when a leaf is illuminated by its adaxial face, the palisade cells oriented perpendicularly to the surface act as a light guide that restricts light-scattering. Thus, one expects a large fraction of the incident light to be transmitted through this first layer of cells. When the light penetrates the spongy mesophyll cells located closer to the abaxial face the efficient light scattering increases the optical path and thus the efficiency of light trapping. This increase would compensate the decrease in light intensity available at the level of the second layer of cells (spongy mesophyll). Thus, when illuminating a leaf by its adaxial face, the rate constant ki_{PSII} would be rather homogenous within the leaf, as shown in Fig. 2. Conversely, when illuminating the leaf by its abaxial face, light would be immediately scattered in the spongy mesophyll cells. This would strongly decrease the light intensity that reaches the palisade cells.

2.4. A comparison between fluorescence induction curves from leaves and isolated thylakoids

As shown above, a strongly absorbed wavelength results in a strong distortion of the fluorescence time course that likely witnesses the widespread distribution of photochemical rates. After having discussed the merits of the present set-up in terms of excitation homogeneity combined to a wide range of accessible intensities, we now turn to the discussion of some of the information that fluorescence induction curves may provide. First, we will address the origin of the pronounced plateau, observed upon illumination under non-saturating conditions of a dark-adapted leaf, in the light of a comparison between leaves and isolated thylakoids. In a previous paper, Joliot and Joliot ascribed this plateau to the retardation in the reduction of the PS II acceptor resulting from the reduction of the PS I acceptors [20]. This assignment was based on the observations that the plateau was less pronounced in thylakoids, which lack PS I acceptors, than in whole leaves and that, the reoxidation of all the electron acceptors downstream of PS II after their photo-induced reduction was biphasic, with the fast phase (completed in ~2 s) being concomitant with the reoxidation of PS I acceptors.

Fluorescence changes resulting from the illumination of a young spinach leaf has been analyzed after infiltration with 150 mM sorbitol (to avoid the osmotic shock that would result from the infiltration with water) and compared to those obtained with thylakoids prepared from the same batch of leaves (Fig. 6). Uncouplers were also added to the leaf infiltration medium in order to collapse the transmembrane electrochemical potential that would result from the hydrolysis of stromal ATP by the CF_0-F_1 ATPase. With dark-adapted leaves, a fast rising phase of small amplitude is followed by a pronounced plateau, itself followed by subsequent fluorescence rise. As will be discussed later, addition of uncouplers induced a pronounced decrease of the amplitude of the first rising phase of the fluorescence time course (Fig. 6A). Yet, it did not affect the plateau whose origin will be now discussed.

The light intensity has been tuned to get equal k_{PSII} values for the leaf and thylakoids. This has been achieved by measuring the fluorescence kinetics in the presence of DCMU (see the inset in Fig. 6B). Since the PS II photochemical rate is proportional to the fluorescence yield [21], the area above the fluorescence induction curve is proportional to the number of available (i.e. oxidized) PS II electron acceptor. The ratio between the area above the curves found with leaves and thylakoids indicates that the total amount of PS II electron acceptors is 1.5 larger in leaves than in thylakoids. Since the soluble electron acceptors located downstream of PS I in the photosynthetic chain are lost during the preparation of thylakoids, these additional electron acceptors found in leaves with respect to thylakoids are likely to be these soluble electron carriers, likely ferredoxin, FNR or NADP. In thylakoids, the pool of PS II electron acceptors has been estimated to ~15 electron equivalents (12 in the PQ pool, one in Q_A plus the 2 iron–sulfur clusters F_AF_B bound to PS I) yielding a pool of 15 1.5=22 electron acceptors in whole leaves. In dark-adapted leaves, the amount of soluble electron carriers can thus be estimated to ~7. The fact that, beyond the plateau, the fluorescence induction curves follow similar time course in thylakoids and whole leaves shows that, the reduction of the soluble PS I acceptors precedes the reduction of the PQ pool and that the size of the PQ pool is similar in the leaf and thylakoids. Strasser and coworkers previously proposed that the redox state of the PS I electron acceptors modulates the overall fluorescence yield. According to their findings, under saturating light excitation the reduction of the plastoquinone pool precedes that of the PS I electron acceptors, as expected if the oxidation plastoquinol at the Q_O site of cytochrome b_6f is the limiting step under strong illumination [22]. Interestingly, the fluorescence kinetics displayed a plateau of similar duration when fluorescence was detected in the far-red or at 680 nm (Fig. 2A) suggesting that the ratio between soluble PS I acceptors and PS II is rather homogenous within the leaf. We note that the extent

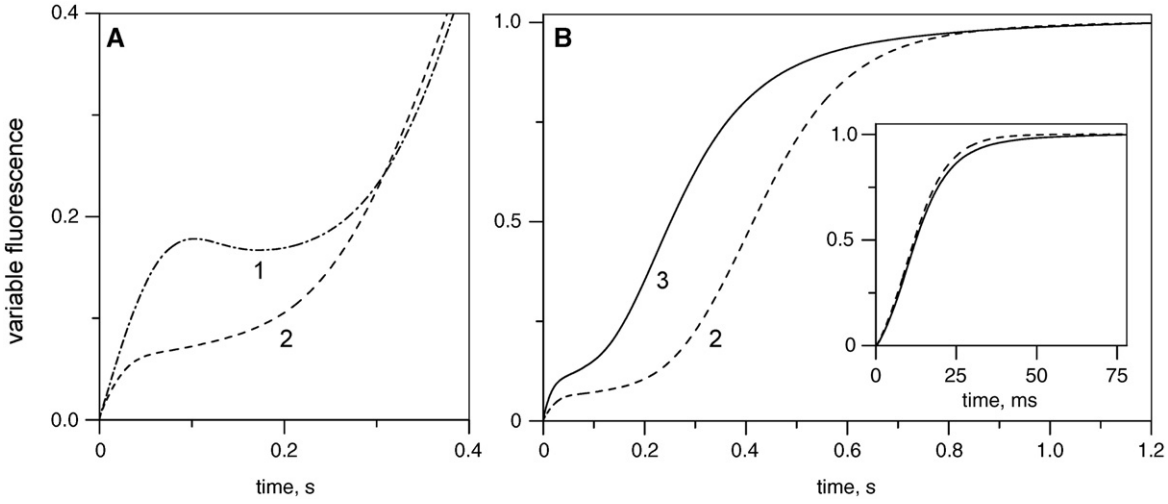

Fig. 6. Fluorescence time course from young spinach leaves or broken chloroplasts. (A) A zoom on the fast initial fluorescence changes. Curve 1: the leaf was infiltrated with 150 mM sorbitol. Curve 2: the leaf was infiltrated with 150 mM sorbitol and 2 μM nonactin, 2 μM nigericin to collapse any permanent transmembrane electrochemical potential. The variable fluorescence yields were normalized to the maximum fluorescence yield measured after a 200-ms pulse of saturating light. (B) Same as panel A Curve 2: fluorescence time course in the presence of uncouplers (same as curve 2 Fig. 5A). Curve 3: fluorescence time course from broken chloroplast isolated from the same batch of leaves than that used in curve 3 (the thylakoids were resuspended in 300 mM sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM HEPES pH 7.2 and 2 μM nonactin, 2 μM nigericin). Insert: fluorescence increase measured in the presence of DCMU. Solid line: thylakoids in the presence of 40 μM DCMU. Dashed line: leaf infiltrated with 150 mM sorbitol, 2 μM nonactin, 2 μM nigericin and 40 μM DCMU.

of the plateau was significantly variable from one leaf to another as reported in [23]. As a trend, mature spinach leaves displayed a larger plateau than young leaves (not shown) suggesting, in the present framework, that the available amount of soluble PS I acceptors depends on the developmental stage of the leaf. As discussed above, such information, which relies on the accurate resolution of the plateau, would be blurred by the use of a strongly absorbed light as an excitation source.

Curves 1 and 2 in Fig. 6A display the initial induction kinetics in the absence or presence of uncouplers, respectively. Addition of uncouplers induces a 3-fold decrease of the amplitude of the first fluorescence rise phase. This effect of uncoupler agrees with previous findings by Diner and Joliot who showed that the fluorescence yield increases with the transmembrane electrochemical potential [24]. In the case of thylakoids under weak light excitation, the first increasing phase has been shown to be mainly associated with the formation of the S_2 and S_3 states of the Water Oxidizing Complex [25–27]. This is illustrated in Fig. 7 (curve 1) in which a non-infiltrated dark-adapted leaf was illuminated by a series of saturating laser flashes 100 ms apart. When measured 20 μs or 99 ms after each flash, the fluorescence yield displays period-4 oscillations in a similar way than those observed with thylakoids [25–28]. Whereas at short times the fluorescence yield is lower in the presence of the S_2 and S_3 states reflecting the increase of the fraction of P^+_{680} presents in the μs time domain with the S-states [16,17,29]; at long times the fluorescence yields are larger with the higher S-states. Indeed, as shown in Fig. 7, curve 1, under these flash excitation-conditions, which are equivalent to a weak illumination (10 charge separations /s), most of the fluorescence increase is associated with the formation of S_2 and S_3 with a minor contribution being associated with the formation of a membrane potential. If one assumes that, under all likelihood, most of the PS II centers are, in the dark, in the S_1 state, the fast initial fluorescence increase observed in the presence of uncouplers is expected to be close to a one quantum process. This provides an alternative experimental mean to calibrate the light intensity in terms of number of absorbed quantum per unit of time. In the case of curve 2 Fig. 6, the time constant of this initial component was ~20 ms, yielding a photochemical rate $k_{PSII} \sim 50\ s^{-1}$.

The fast initial increasing phase was found significantly larger in the case of thylakoids than in the case of spinach leaves in the presence of uncouplers (Fig. 6B). In the case of thylakoids, Lavergne and Leci [25] proposed that the initial

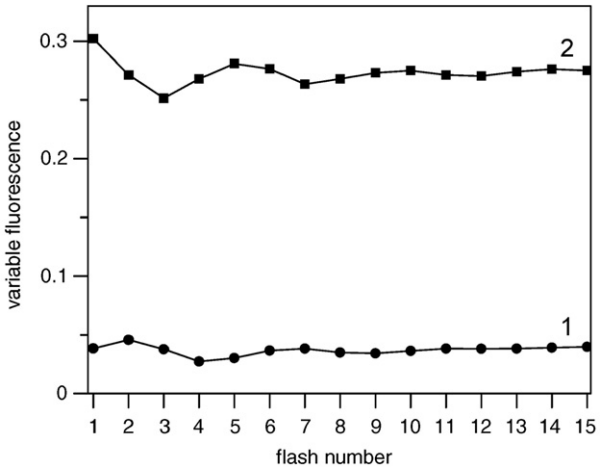

Fig. 7. Fluorescence yield changes from a young spinach leaf submitted to illumination by a flash series. The time interval between flashes was 1 s. Curve 1: fluorescence yield measured by weak green detecting flashes (10 μs duration) 99 ms after each actinic flash. Curve 2: fluorescence yield measured by weak green detecting flashes 20 μs after each actinic flash.

rise of fluorescence induction reflects both the modulation of the fluorescence yield by the S-states and the closure of PS II centers with an impaired electron transfer from the primary quinone Q_A to the secondary quinone Q_B (see [30] for a discussion). These centers (so called inactive centers, [31,32]) would act as DCMU-inhibited centers and would thus give rise to a fast increase in the fluorescence yield upon illumination. Assuming that the modulation of the fluorescence yield by the S-states is similar in thylakoids and whole leaves, the larger amplitude of this initial rise in thylakoids would reflect a larger amount of inactive centers. Yet, in whole leaves, most of the amplitude of this fast initial fluorescence rise can be accounted for by the combined effects of the transmembrane electrochemical potential and S-state increment so that, at variance with *Chlorella sorokiniana* or *Chlamydomonas reinhardtii* cells [25], whole leaves seem to contain negligible amount of inactive centers.

2.5. Measurement of the F_V/F_0 ratio.

Fluorescence time courses are most commonly used to characterize the so-called Φ_{PSII} parameter that corresponds to the electron flux produced by PS II under steady state conditions. This is essentially based on the seminal finding that the fluorescence yield is linearly related to this flux [21]. Thus, if we note F the fluorescence yield at a given time after the onset of the illumination, Φ_{PSII} could be given by the ratio $(F_{max}-F)/(F_{max}-F_0)$. However, two different difficulties are encountered when using this approach. First, the maximum fluorescence yield has been known for long to strongly depend on the light intensity and duration of the illumination owing to non-photochemical quenching processes that develops as a response to illumination [33–35]. This is illustrated in Fig. 8 which shows the fluorescence time course induced by a continuous illumination (200). The F_{max} level was assessed by the superimposition of 200-ms pulse (5500 μE s^{-1} m^{-2}) to the continuous light.

This issue was circumvented by Genty et al. who generalized the above formula [36], assuming that exciton can freely migrate between photosynthetic units: if one notes F'_{max}, the maximum fluorescence yield at a given time and F the fluorescence level induced by the continuous illumination, at the same time, then according to Genty et al. $\Phi_{PSII}=(F'_{max}-F)/F'_{max}$. The second difficulty comes from the implicit underlying assumption in this formula that the PS I fluorescence yield is negligible when compared to fluorescence yield of PS II. This assumption may be questioned since Lavergne and Trissl have estimated that the PS I fluorescence accounts for 25% of the F_0 level [37]. We attempted to experimentally determine the relative contribution of both photosystems in the overall fluorescence. Dark-adapted leaves were illuminated with a 200 ms pulse (k_{PSII}) in order to reach the F_{max} level. The fluorescence was detected through a 680 nm interference filter (FWHM 10 nm) or a high-pass filter (half transmission at 750 nm) in order to selectively cancel or enhance, respectively, the contribution of the PS I fluorescence. The results were compared to those obtained with a high pass band filter (half transmission at 670 nm) which allows detection of the

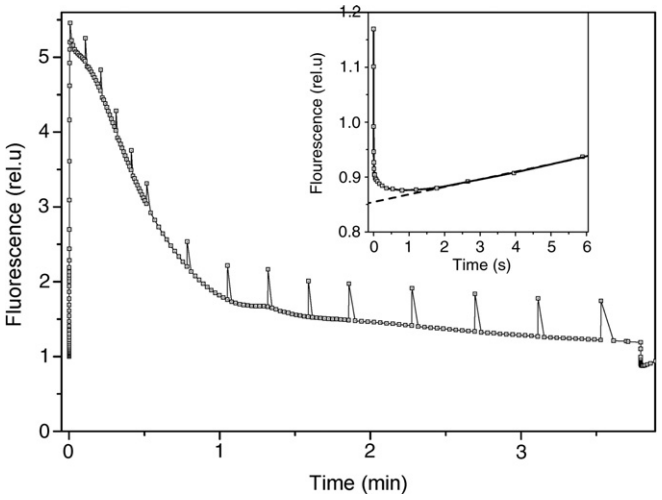

Fig. 8. Fluorescence yield time course during a continuous illumination. Saturating pulses of 200-ms duration were superimposed to the weak continuous illumination to assess the F_{max} level. The inset shows the changes in fluorescence yield after the light was switched off. The fluorescence yield was probed by short (200-μs duration) and weak (400 μE m^{-2} s^{-1} i.e. k_{PSII} ~ 30 s^{-1}) pulses of green light at discrete times after the light was switched off. Under such conditions, the average light intensity is 0.006 photon per second (0.8 μE m^{-2} s^{-1}) so that the actinic effect resulting from probing the fluorescence yield is negligible.

combination of the PS I and PS II fluorescence. Whereas the kinetics of fluorescence changes were identical in the three cases, the F_V/F_0 ratio was significantly different: 6.2, 4.13, and 4.46 when detecting at 680 nm, in the far red and in the red respectively. The high F_V/F_0 level measured at 680 nm is explained by the low F_0 level due to the lack PS I of fluorescence emission at this wavelength [38]. Assuming that there is no other contribution to the fluorescence yield than that from PS II when fluorescence is detected at 680 nm, one can compute that 28% and 34% of the F_0 level does not result from PS II fluorescence when the fluorescence is detected in the red and far red, respectively. As proposed by Lavergne and Trissl [37] PS I is likely to contribute for most of this additional fluorescence, although aggregation of LHCII, which has been observed *in vitro* (see e.g. [39] or [40] for a review) so that their contribution *in vivo* cannot be excluded, is also known to shift the fluorescence emission to larger wavelength. In the following we will adopt a figure of 30% for the contribution of "non PS II fluorescence" to the overall fluorescence yield. From the data in Fig. 8, the F_{max} level reached before the occurrence of NPQ processes is ~ 5.5, yielding a value of $\Phi_{PSII}=4.5/5.5=82\%$ for a dark adapted leaf. Taking into account the PS I fluorescence contribution yields $\Phi_{PSII}=6.2/7.2=86\%$, a figure which is in the range estimated in [41], indicating that neglecting the PS I fluorescence results in a slight, but significant, underestimation of Φ_{PSII}. Yet, if we assume, in agreement with the common thinking, that NPQ processes only affect PS II fluorescence, this underestimation may become more important when, owing to NPQ processes, the relative weight of the PS I fluorescence increases as that of the PS II fluorescence decreases owing to NPQ. As shown in Fig. 8 the F_{max} level may decrease dramatically after a few minutes of illumination. In the present

case the value found for F'_{max} after 3.5 min is 1.74. The question raised by the strong quenching is the extent to which it also decreases $\Phi_{PSII.}$ To address this issue, one needs to determine the F'_0 level. To this aim, we measured the changes in the fluorescence yield after the continuous exciting-light was switched off. As shown in the inset of Fig. 8, the fluorescence yield decreases steeply likely indicating the reoxidation of the PQ pool. In agreement with previous reports [42, 43], it then smoothly increases in the second time range, a fluorescence increase that has been ascribed to the non photochemical reinjection of electron into the PQ pool via the chlororespiratory pathway [44–46]. The extrapolation to $t=0$ yields $F'_0=0.85$. Thus $\Phi_{PSII}=(1.74–0.85)/1.74=.51\%$ or, after correction for the contribution of PS I to the overall emitted fluorescence $\Phi_{PSII}=(1.74–0.85)/(1.74–0.30)=62\%$, to be compared with 86% in dark-adapted leaves. Various mechanisms could account for such a decrease, among which the trapping and dissipation of part of the excitonic energy by the antenna that could occur at the expense of the photochemical trapping by PS II. In this case, one may consider three possible competing pathways for exciton decay: fluorescence, photochemical quenching, and non photochemical quenching with probability k_F, k_{PQ} and k_{NPQ}, respectively. Assuming with Genty et al. [36], that the Stern–Volmer approach applies: $F=k_F/(k_{PQ}+k_F+k_{NPQ})$. The F_{max} and F_0 levels respectively correspond to $k_{PQ}=0$ and $k_{PQ}=1$. Thus $k_F=F_0/(F_{max}-F_0)$ and, assuming k_F and k_{PQ} are constant, $k_{NPQ}=F_0/F'_{max}\cdot(F_{max}-F'_{max})/(F_{max}-F_0)$. Combining theses different equations allows one to calculate $F'_0=k_F/(1+k_F+k_{NPQ})=0.53$ when the various fluorescence levels are corrected for PS I contribution. The good agreement between this value and the experimental one (0.85–0.30=0.55) suggests that, although other factors such as the transmembrane electric field [24,47] generated by the illumination or the inhibition of PS II by acidification of the lumen (see [48] for a review) may contribute to the modulation of Φ_{PSII}, the competition between photochemical and non-photochemical trapping accounts, under the condition of the present experiments, for most of the decrease in Φ_{PSII}.

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