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Excitation kinetics of chlorophyll fluorescence during light-induced greening and establishment of photosynthetic activity of barley seedlings

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Abstract

Excitation kinetics based on feedback regulation of chlorophyll (Chl) fluorescence of leaves measured with the chlorophyll fluorometer, *FluoroMeter Modul* (FMM), are presented. These kinetics showed the variation of excitation light (laser power, LP) regulated by the feedback mechanism of the FMM, an intelligent Chl fluorometer with embedded computer, which maintains the fluorescence response constant during the 300-s transient between the dark- and light-adapted state of photosynthesis. The excitation kinetics exhibited a rise of LP with different time constants and fluctuations leading to a type of steady state. The variation of excitation kinetics were demonstrated using the example of primary leaves of etiolated barley seedlings (*Hordeum vulgare* L. cv. Barke) during 48 h of greening in the light with gradual accumulation of Chl and development of photosynthetic activity. The excitation kinetics showed a fast rise followed by a short plateau at *ca*. 30 s and finally a slow constant increase up to 300 s. Only in the case of 2 h of greening in the light, the curve reached a stable steady state after 75 s followed by a slight decline. The final LP value (at 300 s of illumination) increased up to 12 h of greening and decreased with longer greening times. The active feedback mechanism of the FMM adjusted the excitation light during the measurement to the actual photosynthetic capacity of the individual leaf sample. In this way, the illumination with excessive light was avoided. The novel excitation kinetics can be used to characterize health, stress, disease, and/or product quality of plant material.

Additional key words: chlorophyll fluorescence; embedded system; induction kinetics; intelligent sensors; Kautsky effect; light adaptation.

Introduction

Intact leaves illuminated with actinic light after a dark adaptation of at least 20 min exhibit a change of fluorescence emission of the fluorophore, Chl *a*, which is called 'Kautsky effect' (Kautsky and Hirsch 1931, Govindjee 2004). This fluorescence induction kinetics (or transient) is caused by the change of the fluorescence yield with the onset of various processes of photosynthesis during the dark-to-light transfer induced by the saturating light, which simultaneously excites Chl fluorescence (*see* the comprehensive reviews of the book of Papageorgiou and Govindjee 2004). Induction kinetics of the Chl fluorescence is a nondestructive measurement for many aspects of basic and applied photosynthesis research (*e.g.* Strasser *et al.* 2004).

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Abbreviations: Chl – chlorophyll; F_{690} – fluorescence intensity at the red maximum of the chlorophyll fluorescence emission spectrum of a green leaf near 690 nm; F_{735} – fluorescence intensity at the far-red maximum of the chlorophyll fluorescence emission spectrum of a green leaf near 735 nm; F_0 – minimum fluorescence at the onset of illumination at the leaf dark-adapted state; F_m – maximum fluorescence reached at the leaf dark-adapted state during the first second of illumination with excitation light of saturating intensity; F_p – maximum fluorescence reached during the first second of illumination with the preset initial laser power LP_0 (here: 20% of LP_{max}); F_s – steady state reached at the leaf light-adapted state; F_v – variable fluorescence (maximum fluorescence F_p or F_m minus F_0); FMM – *FluoroMeter Module*; LED – light emitting diode; LP – laser power of the laser diode; LP_0 – preset initial laser power kept constant during the first second of illumination (= LP at the start of the feedback regulation); LP_e – laser power reached after 300 s of illumination; LP_{max} – maximum laser power of the FMM; PAM – pulse amplitude modulation; PAR – photosynthetically active radiation; PSI – photosystem I; PSII – photosystem II.
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With low light intensity the photosynthetic activity rises linearly but becomes saturated with strong light (except for C_4 plants) (*e.g.* Björkman 1981, Larcher 2003). The light-saturation point (*i.e.* the lowest light intensity with which the maximum of photosynthesis is reached) depends on the light conditions of the leaf during its growth and development. Leaves grown in the shade have a light-saturation point below 500 μmol(PAR-quantum) m^{-2} s^{-1}, whereas leaves exposed to full sunlight reach light saturation only at fluence rates above 1,000 μmol(PAR-quantum) m^{-2} s^{-1} (Larcher 2003). Illumination with extreme light intensities, which the leaf has never experienced before, can cause photoinhibition, which is measured as decrease of photosynthetic activity and Chl fluorescence (Long *et al.* 1994) as well as increase of non-radiative de-excitation, *i.e.* the heat production (Buschmann 1987).

For measuring induction kinetics of Chl fluorescence, conventional fluorometers use the same continuous measuring light (adjusted before the measurement) for all samples irrespective of their growth conditions (Strasser *et al.* 2004), *i.e.* the same quantum fluence rate is applied for leaves adapted to high or low light intensities. Many Chl fluorometers use one light source both for exciting fluorescence and inducing photosynthetic activity. However, in measurements with pulse amplitude modulation (PAM), three types of light are applied (for details *see* the review of Schreiber 2004): (*1*) low intensity measuring light [< 0.1 μmol(PAR-quantum) m^{-2} s^{-1}], which excites fluorescence but does not induce photosynthesis, (*2*) medium intensity light ['actinic light', *ca.* 300 μmol(PAR-quantum) m^{-2} s^{-1}] used for inducing photosynthetic activity, and (*3*) 'saturating flashes' applied before and during the illumination together with actinic light for saturating photosynthesis. These flashes are often applied with a quantum fluence rate several times higher than natural sunlight [~ 2,000 μmol(PAR-quantum) m^{-2} s^{-1}, *e.g.* Karageorgou *et al.* 2007] which – depending on the leaf type – exceeds the capacity of the leaf and thus may cause photoinactivation (Apostol *et al.* 2001).

Only in a few studies a nonconstant light is used for the induction of photosynthesis. One protocol is increasing the intensity of the actinic light in several fixed steps. The sequence of these sudden rises of quanta fluence rate takes a few minutes and it is usually the same independent of the light habitat, in which the leaf had been growing. These so-called 'rapid light curves' were introduced by White and Critchley (1999) and later on applied for different research aims (Rascher *et al.* 2000, Ralph and Gademann 2005). They are able to study the response of leaves to increasing irradiance intensity, which is applied in always the same preset fixed steps of light. Each step takes several seconds without waiting to achieve steady state conditions. A dynamic model for describing the electron transport during rapid light curves has recently been proposed by Guarini and Moritz (2009). Nedbal and Březina (2002) also introduced an application of varying light intensity ('harmonic irradiation') for judging the light adaptation of a photosynthetic organism. They concluded that a negative feedback regulation of the plant is responsible for nonlinear modulation of photosynthetic activity (Nedbal *et al.* 2003).

Recently, we presented the technical details of a new type of Chl fluorometer, the *FluoroMeter Modul* (FMM; FMM-4W1, OPTI-SENS, Budapest, Hungary; Barócsi *et al.* 2009). This instrument can measure the conventional induction kinetics of Chl fluorescence ("Kautsky effect"), but it is also able to adjust the intensity to the individual leaf sample by continuously varying the quantum flux rate of the excitation light in such a way that the fluorescence signal is kept constant. This is achieved by a fine regulation feedback adjustment of the laser diode used for fluorescence excitation. The resulting transient between the dark-adapted to the light-adapted state of photosynthesis induced by the laser power (LP) is termed here 'excitation kinetics'. In this way, the intensity of the excitation light is adapted to the actual need and capacity for quantum conversion of an individual sample and is not applied with exaggeratedly high quantum flux rate or in a small number of fixed steps. When keeping the fluorescence constant by the feedback mechanism of the FMM, the change of fluorescence yield during the dark-to-light transfer is compensated by an inverse change of the excitation light.

In this study, we present excitation kinetics measured for primary leaves of barley with a wide range of different Chl content and photosynthetic activity showing their individual physiological plasticity. Barley seedlings, which were grown in the dark for 5 days, were measured during subsequent greening in the light. During the greening process of 48 h of continuous illumination, yellow leaves with no photosynthetic activity turned to fully green leaves with high photosynthetic activity (Baker and Butler 1976, Buschmann 1981).

Materials and methods

Plant material: Barley seedlings (*Hordeum vulgare* L. cv. Barke) were grown at room temperature and 65% relative humidity from the seeds in total darkness on gauze using tap water. Five-day-old seedlings were transferred to continuous white light [compact fluorescent light bulb: *Dulux EL Concentra*, 20 W/*41-827, Osram*, Munich, Germany, 90 μmol(PAR-quantum) m^{-2} s^{-1}]. Measurements were carried out after 2, 4, 8, 12, 24, 36, and 48 h of greening in the light.

Photosynthetic pigments: The total Chl content was monitored *in vivo* using a chlorophyllmeter (*SPAD-502*,

Minolta, Ahrensburg, Germany). The chlorophyllmeter measurements were carried out at the same site as the fluorescence measurements (12 mm below the leaf tip). To exactly quantify the concentration of Chls and carotenoids, pigments were extracted from the same leaf tissue as had been used for SPAD and fluorescence measurements (13 mm length, 5 mm below the leaf tip). Samples were homogenized in 100% acetone and the extract was centrifuged for 300 s at 3,000 × *g* in order to exclude turbidity, which would otherwise disturb the absorption measurement. The quantification of leaf pigments was carried out using a UV-Vis spectrometer (*UV-2101PC*, *Shimadzu*, Düsseldorf, Germany) and the equations according to Lichtenthaler (1987).

Fluorescence measurements were carried out with the Chl fluorometer FMM (*FluoroMeter Modul*, *FMM-4W1*, *OPTI-SENS*, Budapest, Hungary) described in detail previously (Barócsi *et al.* 2009). The FMM, which was already used by Solti *et al.* (2011), is based on a two-wavelength Chl fluorometer (CFM; *CFM-636973, OPTI-SENS*, Budapest, Hungary) established earlier (Barócsi *et al.* 2000, 2003) without feedback regulation and it has been successfully applied by Richter *et al.* (1998) and Georgieva *et al.* (2008). The FMM is equipped with a three-branch optical fiber and a sample holder. The FMM is constructed as a module system, which can be adapted to the particular needs of the measurement (differences in excitation light and/or fluorescence bands, addition of global positioning or watertight setup). The light source for exciting the fluorescence is a 635 nm laser diode (*DL-4038-021*, *Sanyo*, Moriguchi, Japan; maximum optical power: 10 mW). The detection of fluorescence is carried out with two low-noise PIN photodetectors (*SD-200-14-21-241*, *Laser Components*, Hudson, USA) separately for the Chl fluorescence bands at 690 and 735 nm.

For the fluorescence measurement, 13 mm long pieces were cut from the primary leaves about 5 mm below the leaf tip and fixed in the sample holder. The fluorescence was detected at the adaxial (upper) leaf side. The dark adaptation before the fluorescence measurement was at least 20 min. The photosynthetic quantum yield was estimated by calculating the ratio of $F_v/F_p = (F_p - F_0)/F_p$, detected with the 690 nm fluorescence band in the first second of illumination using 20% of the maximum laser power. F_0 and F_p represent the fluorescence intensity at the onset and the maximum of the kinetics, respectively (*see* Fig. 1). The ratio F_v/F_p is equivalent to the often used 'maximum quantum yield of photosystem II', F_v/F_m (Kitajima and Butler 1975), which is calculated with F_m, *i.e.* the fluorescence measured with saturating light. The ratio F_{s690}/F_{s735} was measured after 300 s of illumination in the usual steady state fluorescence F_s.

The FMM enables to measure novel excitation kinetics by adjusting the power of the laser diode (laser power LP) to maintain the fluorescence constant. The maximum laser power (LP_{max} = 100% LP) available at the leaf surface is ~5.6 mW, corresponding to 775 µmol (PAR-quantum) m^{-2} s^{-1}. The recording starts with a preset (initial) laser power (LP_0), which is kept constant within the 1st s of illumination. We chose 20% of LP_{max} in order to have the highest dynamic without reaching LP_{max} within the 300-s measuring period. Thereafter, the FMM system starts changing LP to keep the fluorescence at the level reached at 1 s of illumination. The LP can be adjusted in 256 steps. The increase of the LP correlated very well (r^2 = 0.999, data not shown) with the increase of fluorescence, which was verified using a fluorescence standard (blue plastic film glued on a reflective metal foil: *Rosco Urban Blue 81*, *Rosco Laboratories*, Stamford, USA). The feedback regulation is carried out at each measuring point and LP is changed depending on the needs by only a single step up or down. One of the two fluorescence bands (690 or 735 nm) can be selected as reference fluorescence, which is kept constant.

Data processing of excitation kinetics: The FMM measures and stores the values for the Chl fluorescence at 690 nm (F_{690}) and at 735 nm (F_{735}) as well as for the applied LP with a quasi logarithmic time scale (*i.e.* every 0.1 ms up to 4 ms, and then every 1 ms up to 1 s, every 20 ms up to 15 s, every 200 ms up to 300 s). For a measurement of 300 s, this resulted in 3,161 data points for F_{690}, F_{735} and LP, respectively. The fluorescence amplitudes are stored in the range between 0 and 4,095 units, and the data for LP between 0 and 255 units. In order to characterize the shape of the excitation kinetics, the first derivative was calculated. For this purpose, the data set was reduced to an interval of 0.2 s and smoothed by simplified least square procedure according to Savitzky and Golay (1964) with a broad window of 50 data points (equivalent to an interval of 10 s). The calculations and graphs were carried out using data analysis and graphic software (*Origin 8*, *OriginLab*, Newshampton, USA).

Results

Development of the barley seedlings during greening in the light: After five days of growth in the dark, the barley seedlings reached a shoot length of about 55 mm (excluding seeds and roots). During the following 48 h of greening in the light, this resulted in lengthening of the leaf blade by *ca.* 60 mm (Table 1). The light treatment also led to uncoiling of the primary leaf and to a gradual change of the leaf colour from yellow to fully green. The increase of Chl accumulation in the light could be monitored nondestructively by the rise of the SPAD value

Table 1. Development of 5-d-old etiolated barley seedlings (*Hordeum vulgare* L. cv. Barke) during greening in the light. The length of the seedlings was measured excluding the roots. SPAD values were measured at the same site where the fluorescence measurement was carried out. The chlorophyll concentration [mg Chl (*a*+*b*) m^{-2} and ratio Chl *a*/*b*] was analyzed from extracts with 100% acetone. The ratio F_{s690}/F_{s735} was measured after 300 s of illumination (equal to the steady state F_s). The maximum quantum yield F_v/F_p was measured in the 690 nm band during the 1st s of the excitation kinetics with 20% of the laser power maximum as intensity of the excitation light. Mean with SD (n = 6).

Greening in the light [h]	Shoot length [mm]	SPAD value	Chl (*a*+*b*) [mg m^{-2}]	Chl *a*/*b*	F_{s690}/F_{s735}	F_v/F_p (at 690 nm)
2	56 ± 1	4 ± 1	19 ± 3	6.2 ± 2.1	3.3 ± 0.7	0.78 ± 0.01
4	59 ± 1	6 ± 1	48 ± 13	3.6 ± 0.9	2.0 ± 0.4	0.85 ± 0.01
8	78 ± 3	14 ± 1	80 ± 15	3.3 ± 0.6	1.5 ± 0.2	0.89 ± 0.01
12	85 ± 3	16 ± 2	109 ± 38	3.1 ± 0.5	1.3 ± 0.2	0.89 ± 0.01
24	92 ± 4	24 ± 1	148 ± 31	3.2 ± 0.3	1.2 ± 0.2	0.91 ± 0.01
36	104 ± 4	26 ± 1	165 ± 29	3.2 ± 0.2	1.1 ± 0.1	0.92 ± 0.01
48	114 ± 6	30 ± 1	181 ± 27	3.0 ± 0.2	1.3 ± 0.2	0.92 ± 0.01

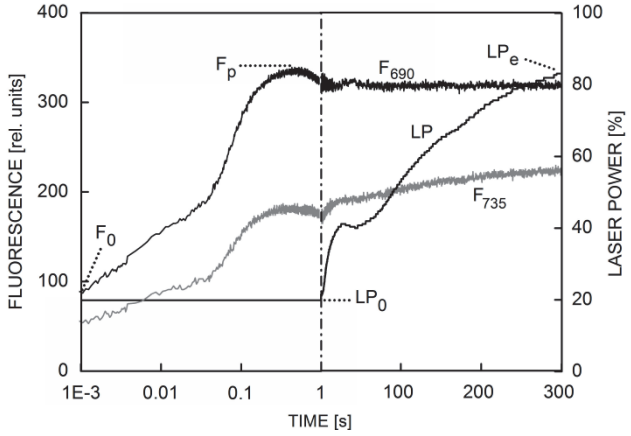

Fig. 1. Example for excitation kinetics of the chlorophyll fluorescence with a fully green, dark-adapted primary leaf of a barley seedling (*Hordeum vulgare* L. cv. Barke, greening in the light for 48 h) measured using the *FluoroMeter Modul* (FMM). It shows the fluorescence in the 690 nm (F_{690}) and 735 nm (F_{735}) bands as well as the laser power (LP) during 300 s of illumination. The LP during the 1st s (LP_0) was set to 20% of the maximum (logarithmic scale); later on (linear scale) it is adjusted by the feedback mechanism to maintain F_{690} constant at the amplitude reached after 1 s. After 300 s LP reaches the final value (LP_e). The fluorescence at the onset of measurement (F_0) and in the maximum during the 1st s of illumination (F_p) are detected to calculate the ratio $F_v/F_p = (F_p - F_0)/F_p$ as a measure of photosynthetic quantum yield.

(Table 1). This value is a direct measure of the increase of the red Chl absorption band, which is corrected for variation in light scattering by a transmittance measurement at 940 nm (*e.g.* Uddling *et al.* 2007). The exact quantification of the leaf pigments by extracts also showed an increase of the total Chl content on a leaf-area basis (Table 1). During 48 h of greening, the carotenoid content was rising (from *ca*. 7 to 30 mg m^{-2}) with a rate slower than that of the Chl content leading to an increase of the ratio Chl to carotenoids from 2.7 to 6.5 (data not shown). Longitudinal growth of leaves, SPAD values, and extracted Chl displayed a parallel rise, which showed that the accumulation of leaf pigments was obviously not yet fully accomplished after 48 h of greening in the light (although the leaves appeared fully green to the eye). The ratio Chl *a* to *b* was the highest after 2 h of greening in the light. Afterwards, the ratio decreased and leveled off at a value of *ca.* 3 after 12 h of greening (Table 1). The greening process could also be followed by the decrease of the ratio F_{s690}/F_{s735} (Table 1), which reached a stable low value after 12 h of greening. During greening in the light, the full photosynthetic activity was already achieved after 8 h of greening according to the fluorescence ratio F_v/F_p (Table 1).

Excitation kinetics at different stages of greening: In Fig. 1, an example for excitation kinetics is given for a fully green leaf (48 h light), dark-adapted for 20 min prior to the measurement. The intensity of the excitation light preselected by the operator was set to 20% of the maximum LP. This initial LP_0 was kept constant throughout the 1st s of illumination. In this time range, the fluorescence rose rapidly from a low value F_0 to a maximum F_p (Fig. 1 *left*, logarithmic time scale). The fluorescence rise was visible for the two bands; at 690 nm (F_{690}) and at 735 nm (F_{735}). The fluorescence signal of one band reached at the 1st s of illumination was then kept constant by the feedback mechanism of the FMM, which regulated the intensity of the excitation light. In the example given in Fig. 1, F_{690} was chosen as the reference signal, which was kept stable during the following 300 s. In the same time, the second band, F_{735}, increased. The feedback regulation resulted in an increase of LP during the light induction curve from the initial value LP_0 to the maximum LP_e reached after 300 s of illumination (Fig. 1 *right*, linear time scale).

Compared to the fluorescence signal of the conventional fluorescence induction kinetics ("Kautsky effect"), the excitation kinetics showed a much slower change of the LP signal. This is clearly demonstrated in Fig. 2 in the comparison of the normalized curves for the excitation

kinetics with the fluorescence induction kinetics using 20 and 100% of LP_{max}.

The excitation kinetics of the primary barley leaves changed strongly during the greening in the light. In Fig. 3, the excitation kinetics are shown as a mean of 7 independent measurements with standard deviation. The variation of the replicates was the highest after 4 h of greening (*see* also Figs. 5, 7*B*). After 2 h of greening in the light, the LP increased only slightly and soon reached a low steady state (Fig. 3). In contrast to the other curves, the LP rise of these yellowish green leaves was relatively fast and even declined after 120 s (*see* the normalized curves in Fig. 4). After 4 h of greening, the rise of LP within the first 15 s was followed by a second less pronounced rise after 75 s. The intermediate level at 30 s was the most pronounced for the leaves greening for 8

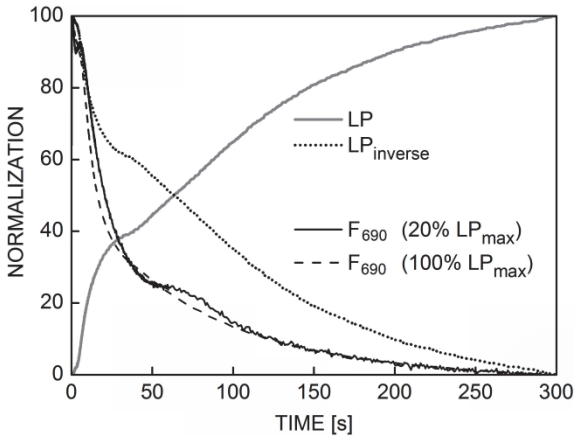

Fig. 2. Excitation kinetics (LP) and its inverse ($LP_{inverse}$), as well as fluorescence induction kinetics at 690 nm (F_{690}) with 20 and 100% LP_{max} for the fully green primary leaves of barley seedlings (*Hordeum vulgare* L. cv. Barke, 5-d-old etiolated seedlings after greening in the light for 48 h). The curves represent the mean of 6 measurements normalized to a maximum spread scaling (minimum – maximum) for the time between 1 and 300 s.

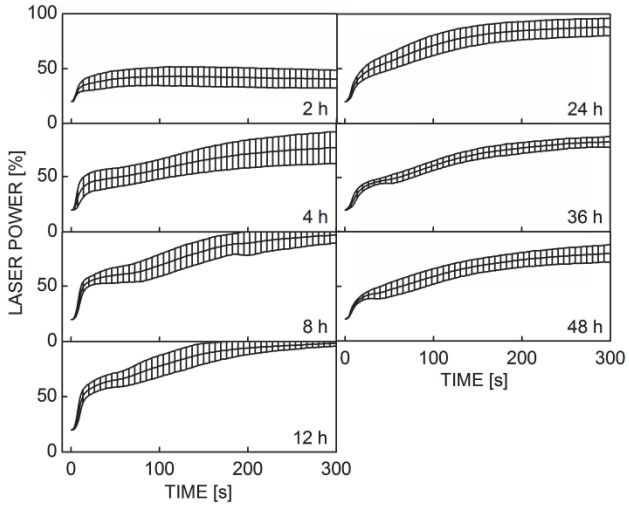

Fig. 3. Excitation kinetics with primary leaves of 5-d-old etiolated barley seedlings (*Hordeum vulgare* L. cv. Barke) after greening in the light for 2, 4, 8, 12, 24, 36, and 48 h. It shows the kinetics of the laser power (LP) between 1 and 300 s of illumination using the *FluoroMeter Modul (*FMM). For each kinetics the initial LP (LP_0) was set to 20% of the maximum, later on it was adjusted by the feedback mechanism of the FMM to maintain the fluorescence band at 690 nm constant at the amplitude reached after 1 s of illumination. The kinetics is the mean of 7 independent measurements with SD.

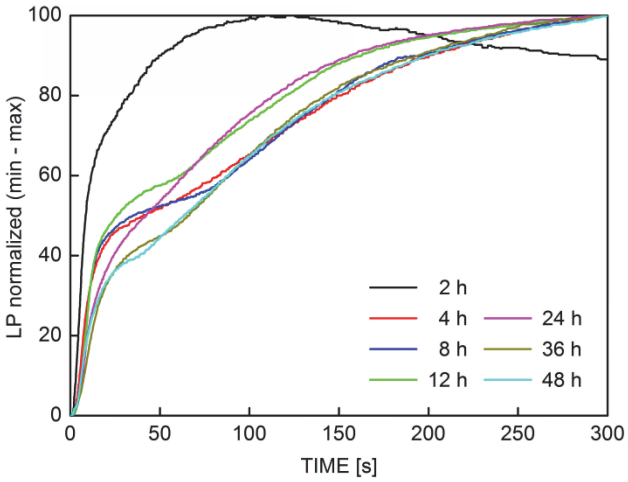

Fig. 4. Normalized excitation kinetics for the primary leaves of 5-d-old etiolated barley seedlings (*Hordeum vulgare* L. cv. Barke) after greening in the light for 2, 4, 8, 12, 24, 36, and 48 h. The mean values of the curves shown in Fig. 3 with maximum spread scaling (minimum – maximum).

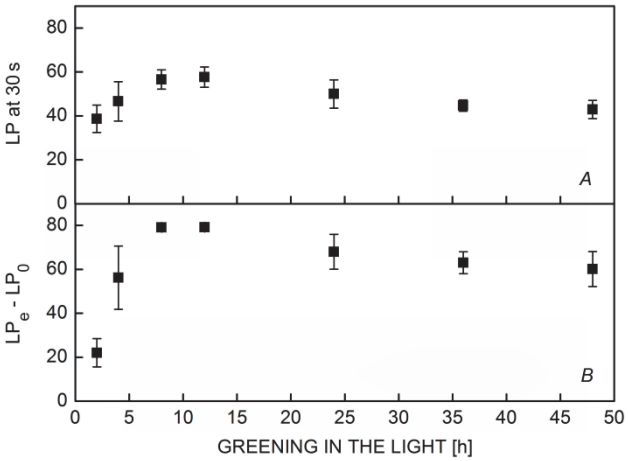

Fig. 5. Values of the laser power (LP) during the excitation kinetics with primary leaves of barley seedlings (*Hordeum vulgare* L. cv. Barke) for different times of greening in the light (mean of 7 measurements with SD). The excitation kinetics (Fig. 3) was measured using the *FluoroMeter Modul* (FMM) with an initial LP of 20% of the maximum. The fluorescence at 690 nm was used as reference band and kept constant by the feedback mechanism of the FMM. (*A*) Laser power at 30 s of illumination, (*B*) Maximum difference of the laser power during the excitation kinetics ($LP_e - LP_0$).

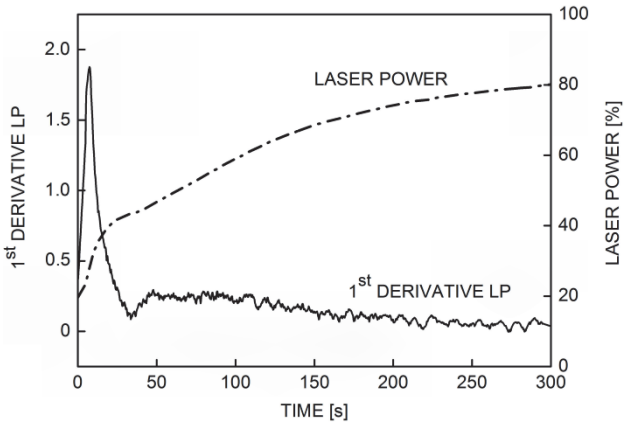

Fig. 6. Example for the excitation kinetics of the laser power (LP) and the resulting 1st derivative measured with a fully green dark-adapted primary leaf of a barley seedling (*Hordeum vulgare* L. cv. Barke, greening in the light for 48 h) using the *FluoroMeter Modul* (FMM). For the kinetics the initial LP (LP_0) was set to 20% of the maximum and the fluorescence at 690 nm was used as reference band which was kept constant by the feedback mechanism of the FMM. The 1st derivative was calculated after smoothing the original curve by simplified least square procedure.

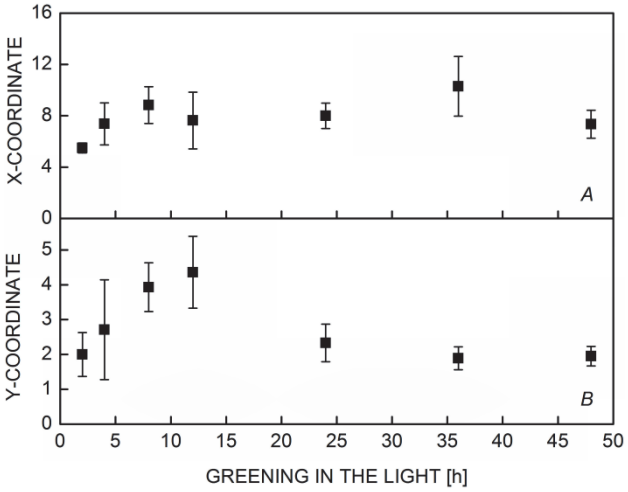

Fig. 7. Maximum of the 1st derivative of the excitation kinetics (Fig. 3) with primary leaves of barley seedlings (*Hordeum vulgare* L. cv. Barke) for different times of greening in the light. The excitation kinetics was measured using the *FluoroMeter Modul* (FMM) with an initial LP of 20% of the maximum. The fluorescence at 690 nm was used as reference band and kept constant by the feedback mechanism of the FMM. The kinetics is the mean of 7 measurements with SD. (*A*) Time at which the maximum rise of the laser power (LP) kinetics was reached (x-coordinate of the maximum of the 1st derivative of the excitation kinetics), (*B*) Amplitude of the maximum rise of the LP kinetics (y-coordinate of the maximum of the 1st derivative of the excitation kinetics).

and 12 h (Fig. 5*A*). In the excitation kinetics recorded after 8 and 12 h of greening, 100% of LP (limited by the laser diodes of the FMM) was sometimes reached before the end of the recording time, which can be seen by standard deviations beyond 100% (Fig. 3). The final maximum LP_e reached after 300 s increased up to 12 h of greening and subsequently decreased slightly with the leaves of the seedlings greening for 24 and 48 h in the light (Fig. 5*B*).

Rise of the excitation kinetics described by the first derivative: In order to analyze the rise of the excitation kinetics, the first derivative was calculated, which represents the change of the slope of the LP curve (*see* example in Fig. 6). The rise of the LP values during the 300 s of feedback regulation was characterized by a maximum reached within the first 10 s of the kinetics.

The time for the highest rise of the LP curve, which was expressed as x-coordinate of the maximum in the first derivative of the excitation kinetics, was the lowest after 2 h of greening (Fig. 7*A*) and showed slight variation at a higher level during the later greening stages. The amplitude of the rise of the LP curve, expressed as y-coordinate of the maximum in the first derivative of the excitation kinetics, was the highest with the leaves of the seedlings greening in the light for 12 h (Fig. 7*B*). It was clearly lower at the beginning of greening (2 h) and also after 24, 36, and 48 h of greening.

Discussion

Fluorescence rise during the 1st s of illumination: The new Chl fluorometer FMM (Barócsi *et al.* 2009) measures the rise of the fluorescence during the 1st s of illumination with a constant excitation light at a user-defined initial laser power (LP_0). The 1st s of the measurement displays the potential capacity of photosynthesis and represents a fluorescence induction kinetics measured with nonsaturated excitation light. The interpretation of fluorescence transients is essentially based on the principle that the redox state of Q_A, the primary electron acceptor of photosystem II (PSII), modulates the fluorescence yield as introduced by Duysens and Sweers (1963). The fluorescence rise to a maximum (peak level or F_p) within the 1st s of illumination is a consequence of the reduction of the redox substances behind the reaction center of PSII. The kinetics maximum, F_p, is interpreted as a transient block of the electron transport (Satoh and Katoh 1980) with minimal photochemical and nonphotochemical quenching. However, for the measurements with the FMM one must take into account that this part of the fluorescence induction kinetics is carried out with low (usually not saturating) intensity of the excitation light

(here: 20% of LP_{max}) and thus causes lower fluorescence intensity with a different kinetics than with saturating light (*e.g.* Strasser *et al.* 1995). The fast rise of the Chl fluorescence is normally used for measuring maximum quantum efficiency as $F_v/F_m = (F_m - F_0)/F_m$ (Kitajima and Butler 1975). In this study, the kinetics maximum was termed F_p in contrast to the usually used term F_m, which should be detected using saturating light. In principal, it is unknown, which intensity is needed to achieve a full saturation of an individual leaf sample. Recently it has been demonstrated that in many cases extreme intensity of light or irradiance is needed for the saturation and with the usually applied nonsaturating intensity, F_v/F_m is underestimated (Karageorgou *et al.* 2007). In our case, the values for F_v/F_m would be higher than those for F_v/F_p (Table 1). The increase of the ratio F_v/F_p during greening in the light was a clear indicator of the increasing photosynthetic activity. F_v/F_p reached a maximum already after 12 h of greening and was constant, when the Chl content on a leaf area basis (determined from the extract) was still increasing up to 48 h of light. The fluorescence at 690 nm reached after 1 s of illumination used as reference value for the feedback mechanism of the FMM can be regarded as equivalent to the maximum of the fluorescence induction kinetics (F_p). Depending on the laser power, F_p was reached slightly earlier than 1 s, *e.g.* when using 20% of LP_{max} (Fig. 1).

Feedback regulation of FMM: The new FMM offers the possibility to measure excitation kinetics of Chl fluorescence. During excitation kinetics the LP rose from the user-defined low initial value (LP_0 = 20% LP_{max}) to the high value at 300 s of illumination (LP_e) (Fig. 1). The excitation kinetics shows the change of LP, when Chl fluorescence is kept constant using the active FMM feedback mechanism. They can also be termed 'excitation kinetics' (Barócsi *et al.* 2009) since they represent a type of 'equal-response curve' used for the extraction of the effective absorption of photoreceptors leading to the action spectra of these photoreceptors (*e.g.* Fork and Amesz 1969). The feedback mechanism of the FMM is fast and sensitive enough to achieve the goal of keeping the fluorescence constant over time at the chosen fluorescence band. Oscillations due to the feedback regulation, which might eventually lead to a collapse of the feedback regulation, have not been observed. The reason for this is that the FMM feedback regulation is carried out very frequently (at each measuring interval, *i.e.* every 20 ms during the first 15 s and every 200 ms later on) and changes of LP are only carried out in one single step up or down at each point of measurement. In cases, when LP reaches the maximum LP_{max} (limited by the maximum power of the laser diode of the FMM), the fluorescence at the chosen reference band is slightly decreasing.

The feedback regulation of the FMM can be carried out by using the Chl fluorescence at 690 nm (F_{690}) or 735 nm (F_{735}) as reference band. The ratio F_{690}/F_{735} can be taken as an indicator of Chl content of the leaf sample (Buschmann 2007). In cases, when F_{690} was kept constant, the F_{735} increased slightly (Fig. 1), whereas with F_{735} as reference band, F_{690} decreased (unpublished data). The different behavior of the two bands is ascribed to the state 1/state 2 transition (Lombard and Strasser 1984). In conventional fluorescence induction kinetics, F_{690} decreases more strongly than F_{735} (Buschmann and Schrey 1981). This can be partially explained by the gradual appearance of photosystem I (PSI) fluorescence in the long wavelength band (Bradbury and Baker 1981, Franck *et al.* 2002) during the induction time, in which the energy transfer is changed from PSII only (state 1, at the onset of illumination) to PSII and PSI (state 2, after several minutes). This is basically due to the differences in absorption of PSI and PSII (*e.g.* Pfündel 2009). The appearance of a long-wavelength fluorescence component related to nonphotochemical quenching has been proposed as a cause for the uneven decrease of the two Chl fluorescence bands (Lambrev *et al*. 2010). Thus, when keeping one fluorescence band constant (as it is done with the FMM), the other band is changing its intensity.

Excitation kinetics presents the change of the light intensity used for inducing the photosynthetic electron transport during the dark-to-light transition. This change is an active process influenced individually by the leaf sample. The feedback mechanism of the FMM reacts to the fluorescence changes of the sample and adjusts the LP individually to the sample. The increase of LP is not just a mirroring of the classical fluorescence induction curve, which is clearly demonstrated in Fig. 2. The decline of the fluorescence induction kinetics (using constant LP) was much faster than the rise of the excitation kinetics. Excitation kinetics do not resemble the classical 'light-saturation curves' taken in the steady state or the 'rapid light curves' taken in intervals of usually 10 s without waiting for steady-state conditions (White and Critchley 1999). Light-saturation curves and rapid light curves are passive types of measurement, which use the same light intensity or steps, irrespective of the individual leaf sample. The feedback regulation of the FMM is also different from the 'fluorescence clamp'-technique of Schinner *et al.* (2000), who studied the variation of the light intensity of LEDs. In this study, the low-intensity pulses of the measuring light of a PAM-instrument (which does not affect the photosynthetic activity) was regulated in order to keep the Chl fluorescence constant. They interpreted their results in terms of fluxes into and out of the antenna pool of PSII.

A feedback regulation adjusting the intensity of the excitation light in order to keep the Chl fluorescence stable is used by an instrument with a dual wavelength excitation (DUALEX) introduced by Goulas *et al.* (2004). This technique is developed to acquire rapid information about the absorption characteristic of the leaf tissue and thus is used to 'fully eliminate any artifact caused by

variable Chl fluorescence' as it has been demonstrated for quality assessment in grape vine production (Cerovic *et al.* 2008). Hence the changes in the excitation light intensity are not considered or stored.

In general, the intensity of fluorescence (F) depends on the intensity of the excitation light (I_0), the absorption of excitation light (A) either directly by the fluorophore or by other pigment molecules, which transfer the absorbed energy to the fluorophore, and the fluorescence yield of the fluorophore (Φ_f) (*e.g.* Dau 1994).

$$F = I_0 \times A \times \Phi_f$$

During the time of Chl fluorescence induction kinetics (in general a few minutes), A can be considered to be constant. F depends on intensity of I_0, and Φ_f. By adjusting the intensity of the excitation light required to maintain a constant fluorescence level, it is possible to monitor individually for each leaf sample the changes of the fluorescence yield during the dark-to-light transition. The change of the fluorescence yield, Φ_f, after onset of illumination shown by the excitation kinetics is a consequence of the increase of photochemical and nonphotochemical quenching processes. These changes are monitored by the transient, which starts from a maximum fluorescence state (F_p) with closed traps of the PSII reaction centers and minimum of quenching. This leads to a steady state (F_s) with an equilibrium between open and closed traps of the PSII reaction centers and an increase of nonphotochemical quenching due to the onset of PSI activity, thylakoid pH gradient, LHC dephosphorylation and Calvin cycle functions (*e.g.* Dau 1994).

In the first seconds of the kinetics (shortly behind the maximum, F_p) the photochemical quenching is normally at a high level and declines afterwards. The nonphotochemical quenching, which is low in the first seconds, rises towards the end of the kinetics (PAM-measurements, data not shown). When LP reached a constant level at the end of the excitation kinetics, the fluorescence (which had the same level as after 1 s of illumination) corresponded to the known steady-state fluorescence (F_s), which would have been detected with the high LP_e that is finally reached by the FMM feedback regulation. At the F_s level, the photochemical quenching is low, on the other hand, nonphotochemical quenching is high.

In contrast to classical fluorescence induction kinetics ("Kautsky effect"), in excitation kinetics the fluorescence after 300 s has the same amplitude as F_p, which is achieved by a gradual increase of the intensity of excitation light. This corresponds to a F_s of the fluorescence induction kinetics with the LP used after 300 s (LP_e). The conventional measurement of fluorescence induction kinetics with a fixed continuous intensity of the actinic (excitation) light do not only disregard the 'history' of light reception of the leaf sample, they also lead to different results of the parameters determined depending on the (constant) intensity of the actinic light applied with different instruments (*e.g.* the parameter q_p is always high with low actinic light, but low with high actinic light).

Generally, there is no rise of the LP when LP_0 is chosen very low (*i.e.* at about the same level as with the measuring light of PAM-fluorometers, when measuring F_0 fluorescence signal), because the low light does not induce photosynthetic activity and no variation of the Chl fluorescence would show up. In addition, no rise of LP should be observed – even with higher LP_0 – when photosynthesis is inhibited. The increase of LP during the excitation kinetics becomes stronger with higher LP_0 chosen at the onset of the measurement (Barócsi *et al.* 2009).

The rise between LP_0 to LP_e was low for the primary leaves of barley after 2 h of greening and increased till 12 h of greening. This can be explained by the rising photosynthetic activity indicated by the increasing values for F_v/F_p (Table 1). The speed of the rise in the first few seconds of the excitation kinetics was also the highest after 12 h of greening; *see* the first derivative of this part (Fig. 7*A*, x-coordinate). A possible explanation was a high rate of electron transport and a high nonphotochemical quenching in this stage of greening. When the leaves were greening in the light for 24 h and more, the higher amount of accumulated Chl needed less LP to maintain a constant fluorescence yield. In these green leaves, the efficiency of energy transfer in the antenna system was improved and less energy was needed to keep the fluorescence constant.

The rise of LP during the excitation kinetics is fast, when the trans-thylakoidal proton gradient and the state 1/state 2 transition are established quickly. This is the case with leaf samples which have been dark adapted only for a few minutes (Barócsi *et al.* 2009). A similar situation was found in this study with leaves containing less Chl, which were greening in the light only for a few hours (Fig. 7*B*, y-coordinate). If the leaves have been dark-adapted for a longer time before the onset of the measurement, the rise of LP is rather slow and in several cases five minutes (the maximum measuring time set by the instrument) are not sufficient to reach a final steady state of LP (Barócsi *et al.* 2009). This resembles the situation of the leaves having accumulated higher amounts of Chl.

The few cases of decrease of LP in the later part of the excitation kinetics could be related to limitation in the activation of ferredoxin-$NADP^+$-reductase (Lazár and Schansker 2009), to a lack of availability of CO_2 (Walker *et al.* 1983), an increase in nonphotochemical quenching (a very general reason with many possible causes, *see e.g.* Lambrev *et al.* 2010) or a gradual inhibition of photosynthesis taking place during the time of the excitation kinetics (*e.g.* penetration of an inhibitor of photosynthesis, Lichtenthaler *et al.* 1997).

Conclusions: Conventional fluorescence induction kinetics using constant light with the same fixed intensity for all leaf samples represents a passive type of measurement. Depending on the sample, the intensity of the excitation light might be too low or too high to initiate all steps of the photosynthetic process at the transient between dark and light properly. The feedback mechanism of the FMM adjusts the excitation light actively during the measurement to the actual photosynthetic capacity of the individual leaf sample. Illumination with excessive light is thus avoided. The excitation kinetics represents an active type of measurement, which shows the physiological flexibility and plasticity of the individual leaf sample and can be also used to judge the effectiveness of light-energy utilization of plants more clearly than with conventional induction kinetics using constant actinic (excitation) light. To fully understand excitation kinetics, a mathematical modeling taking into account the various processes during the induction of photosynthesis would be useful (Laisk *et al.* 2009). But, up to now, for the complex and often species-dependent fluorescence changes of the dark-to-light transition after the 1st s of illumination (*i.e.* the time interval for excitation kinetics), 'a full experimental description of processes involved was never achieved' (Lazár and Schansker 2009).

The new feedback regulation of the excitation light carried out with the FMM would be a valuable tool for other types of fluorometers in scales between microscopy and remote sensing. With a continuous feedback regulation of the PAM-parameter 'effective quantum efficiency' ($\Delta F/F_m'$, Genty *et al.* 1989), one could optimize plant growth and on a large scale reduce energy consumption. Similar feedback regulation mechanisms should be advantageous for other applications with the aim to achieve optimal light conditions, *e.g.* O_2 and CO_2 gas exchange or greenhouse management by an intelligent fluorosensor concept.

In future, excitation kinetics could be applied as a general 'load test' for different realistic light conditions. They can be also used to characterize health, stress, disease, and/or product quality of plant material, they represent an emerging technique for many aspects in basic and applied photosynthesis research.

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