Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*☆

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A new instrument (M-PEA), which measures simultaneously kinetics of prompt fluorescence (PF), delayed fluorescence (DF) and modulated light reflection at 820 nm (MR), was used to screen dark-adapted leaves of the resurrection plant *Haberlea rhodopensis* during their progressive drying, down to 1% relative water content (RWC), and after their re-watering. This is the first investigation using M-PEA, which employs alternations of actinic light (627-nm peak, 5000 μmol photons m−2 s−1) and dark intervals, where PF-MR and DF kinetics are respectively recorded, with the added advantages: (a) all kinetics are recorded with high time resolution (starting from 0.01 ms), (b) the dark intervals’ duration can be as short as 0.1 ms, (c) actinic illumination can be interrupted at different times during the PF transient (recorded up to 300 s), with the earliest interruption at 0.3 ms. Analysis of the simultaneous measurements at different water-content-states of *H. rhodopensis* leaves allowed the comparison and correlation of complementary information on the structure/function of the photosynthetic machinery, which is not destroyed but only inactivated (reversibly) at different degrees; the comparison and correlation helped also to test current interpretations of each signal and advance their understanding. Our results suggest that the desiccation tolerance of the photosynthetic machinery in *H. rhodopensis* is mainly based on mechanism(s) that lead to inactivation of photosystem II reaction centres (transformation to heat sinks), triggered already by a small RWC decrease.

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1. Introduction

The chlorophyll (Chl) a fluorescence emitted by higher plants, algae, lichens and photosynthetic bacteria upon illumination (prompt fluorescence, PF) carries a lot of information for the structure and function of the photosynthetic apparatus. The discovery of the variable fluorescence by Kautsky [1] showed that it consists of a fluorescence rise until a peak P (Fp) and a subsequent decrease until a steady state S (F0). In higher plants and algae the fluorescence emitted at room temperature originates, predominantly, from the antenna Chls of photosystem (PS) II. For several decades, the true extremes of the fast rise, i.e., F0 at the origin O – where all PSII reaction centres (RC) are open, and Fm – the maximal Fp (reached when all RCs are closed), could not be detected due to the poor time resolution and weak illumination, respectively, of the former instruments.

The resolution of the fast fluorescence rise was highly improved with instruments using optoelectronic parts. With modulated light-fluorometers accurate F0 values could be measured using a weak modulated light beam before illumination with actinic light, and Fm could also be reached under the strong illumination used; though the...
The specific energy fluxes (fluxes/RC) are the products of quantum yields (fluxes/ABS) and ABS/RC, experimentally determined as:

\[
\text{ABS} = \frac{\text{TR}_A}{\text{RC}} \quad \text{and} \quad \text{ABS/RC} = \frac{\text{TR}_A/\text{ABS}}{\text{RC}}
\]

\[
\text{RC} = \frac{\text{TR}_A}{\text{ABS}} \quad \text{where} \quad \text{TR}_A = \frac{(\text{DF}_M - \text{DF}_E)}{\text{F}_M - \text{F}_E}
\]

\[
\text{ABS} = \text{TR}_A \times \text{ABS/RC} = \frac{(\text{DF}_M - \text{DF}_E)}{\text{F}_M - \text{F}_E}
\]

The relative variable fluorescence, \(V_t\), is defined as:

\[
V_t = \frac{\text{F}_t - \text{F}_0}{\text{F}_M - \text{F}_0}
\]

The total electron carriers (ECs) per RC is defined as:

\[
\text{EC}_t = \frac{\text{Area}}{\text{RC}}
\]

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**Fig. 1.** A schematic presentation of the JIP-test (modified after [9]). For details see Material and methods section.
hydrated and high resistant when dry and inactive. Experimental approaches based on bioenergetics have been applied to investigate the dynamics of the inactivation of the photosynthetic machinery upon drying and reactivation upon re-watering [12,13]. There are few higher plants that can tolerate drastic water loss, thus termed as poikilohydric or desiccation tolerant or, popularly, as resurrection plants. Depending on the way of “switching off” their photosynthetic apparatus under water loss, they are distinguished in homiochlorophyllous desiccation tolerant (HDT) and poikilochlorophyllous desiccation tolerant (PDT). HDT plants respond with inactivation of the thylakoid system that is preserved in a non-functional, but easily restorable form [14,15], while in PDT plants the chlorophylls and the thylakoid system are completely damaged and need to be entirely reconstructed and revived [16,17].

Haberlea rhodopensis is a rare HDT plant, endemic in Bulgaria; the leaves, photosynthetically fully active at the hydrated state, can undergo drying to an inactive state and be revitalized upon re-watering [15,18,19]. In the present work we screened leaves during their drying down to a relative water content of 1% and at their fully re-watered state (all treatments in darkness to avoid photoinhibition), by measuring simultaneously, with the M-PEA instrument, kinetics of PF, DF and MR. Our work aimed to advance the understanding of the desiccation tolerance strategies of H. rhodopensis and, by comparing the different water-content-states where the photosynthetic machinery is not destroyed but only inactivated (reversibly) at different degrees, to recognize the differences in respect to each of the three signals and their correlation, which would also help to test and advance the current interpretations of each signal.

2. Material and methods

2.1. Plant material

Fresh H. rhodopensis plants, with adjacent soil layer, were collected from their habitat (near Bachkovo in the region of Plovdiv) and transferred to a garden, where they continued to grow under natural conditions and thick shade (under a walnut tree).

Young fully developed leaves from the middle part of the rosette, with approximately the same colour and size, were used in the experiments. After being hydrated until water saturation, by placing them between two layers of wet filter paper, they were put in the sample-clip of M-PEA and the measurements’ protocol described below was applied. The leaves were then dried in the dark, directly in sample-clip of M-PEA and the measurements’ protocol described above was applied. The OFF and ON switching of all emitters and detectors is controlled by a time control unit according to the chosen protocol scheme, drawn.

After each measurement, the relative water content (RWC) was determined by gravimetric method, i.e., by weighing the sample (W) and applying the equation RWC = [(W - Wdry)/(Wsat - Wdry)] *100, where Wdry and Wsat are the sample’s weights at fully dried (in oven at 100 °C) and water-saturated state respectively.

Chlorophyll (Chl) content of H. rhodopensis leaves was determined in parallel experiments, according to [20]. Leaf discs of 10 mm diameter, from the same plant, were numbered and each of them was weighted after being fully hydrated. After different drying times (DT), including DT = 0 (100% RWC), Chl was extracted from each disc separately, after the disc was weighted (so that the RWC could also be determined). The Chl content of each disc was calculated on the basis of the weight of the disc at the fully hydrated state. Three discs were used after each DT and the Chl content values were averaged. The experiment was repeated for two more times; each time the leaf discs were taken from a different haberlea plant.

2.2. Simultaneous measurements of the kinetics of prompt fluorescence (PF), delayed fluorescence and modulated 820 nm reflection (MR) with the Multifunctional Plant Efficiency Analyser M-PEA

The kinetics of prompt chlorophyll (Chl) a fluorescence (PF), delayed Chl a fluorescence (DF) and modulated reflection at 820 nm (MR_820, or simply MR) were simultaneously recorded with the Multifunctional Plant Efficiency Analyser M-PEA (built by Hansatech Instrument Ltd, King’s Lynn, Norfolk, PE30 4NE, UK). Measurements were conducted on dark-adapted (for 1 h) leaves, still attached on the plants (only for the experiment shown in Figs. 2 and 3) or after their above described treatment. Three independent experiments were conducted, each with leaves from a different haberlea plant; the experiments were reproducible and the data from a representative one are here presented. Care was taken so that the measurements performed during progressive dehydration were done on different leaf spots: for each experiment, three leaves were used; one for the measurements after DT = 0–13 h, the second after DT = 10–23 h and the third leaf after DT = 20–34 h.

Since in the present work we refer to two analytical times (and also to the experimental time for the drying treatment of the leaves – drying-time DT), we denoted the analytical time for the PF and MR kinetics as “JIP-time” and the analytical time within each dark interval during which DF is recorded as “delay-time” – counted from the moment that actinic illumination is interrupted. Though what we observed in the dark-intervals were DF decay kinetics, we prefer the term “delay-time” instead of “decay-time,” because it is theoretically possible that some DF components may temporarily increase during this time.

Three emitters and four detectors’ assemblies are built in the M-PEA sensor unit. The emitter wavelengths are 627 ± 10 nm for the actinic light LED, 820 ± 25 nm for the modulated light LED and 735 ± 15 nm for the far-red light LED; the latter uses a RG9 long pass filter to remove any visible light component. High quality optical band pass filters are used for the protection of the detectors of prompt and delayed fluorescence (730 ± 15 nm) and modulated reflection (820 ± 20 nm). The LED emitting in the far-red (735 ± 15 nm; 1000 μmol photons m⁻² s⁻¹ at 100%) can be used when fast re-opening of PSII reaction centres is required (e.g., for samples being at the light-adapted state). Moreover, measurement of the reflection of this beam, in combination with those of the 627, 735 and 820 nm beams (by the fourth, broad spectrum, detector) permits the determination of the relative absorptivity of the leaf (not used in the present study). The actinic light LED is built into the centre of the optical sensor unit and focused onto the sample surface to provide homogeneous illumination over the exposed circular area (2 mm diameter), with an intensity of 5000 μmol photons m⁻² s⁻¹ at 100% (in the present work, the maximal intensity was applied). The other emitters and detectors are built in the periphery of the unit.

The data acquisition for the three signals, PF and MR in the light and DF in the dark, is every 0.01 ms in the digitalization range 1 (0.01–0.3 ms), every 0.1 ms in range 2 (0.3–3 ms range), every 1 ms in range 3 (3–30 ms) and decreases accordingly until range 7 (30–300 s) where the data acquisition is every 10 s (see Table A2 in the Appendix).

The simultaneous measurements of PF and DF require alternation of light and dark intervals, where PF vs. JIP-time is registered in the former and DF vs. delay-time in the latter. The duration of the dark pulses can be set as a fixed time, or as a fraction of the time between two successive PF data acquisition in each JIP-time digitalization range. In range 1 no dark interval was applied and only PF is recorded. The ratio of the duration of light to dark intervals in the ranges 2–7 was set here at about 76% to 24%. During the 0.3–30 ms JIP-time, the three PF data points registered after each dark interval were not used in the present analysis because they are affected by the dark interruption. The protocol is shown in Table A2.

The OFF and ON switching of all emitters and detectors is controlled by a time control unit according to the chosen protocol scheme, drawn
with a special program of M-PEA and loaded in the apparatus memory. The separation of the signals from PF and DF is also accomplished by the time control unit, which switches the signal record between the two detectors. All signals are amplified and digitized by analogue to digital converters (the amplification for DF is about a hundred times that for PF) and registered in the apparatus memory.

From the reflected beam signal MR_{220} (or simply MR; scattered in all directions), the ratio MR/MR_{0}, where MR_{0} is the value at the onset of the actinic illumination (taken at 0.7 ms, the first reliable MR measurement), was calculated. This ratio is the complementary of the fraction \(\frac{\text{abs}_t}{\text{abs}_{220}}\) of incident light flux that is absorbed (\(\text{abs}_t\)) by the sample (at 820 nm). Therefore, a decrease of MR/MR_{0} is equal (in amplitude) with an increase of \(\frac{\text{abs}_t}{\text{abs}_{220}}\). The latter is associated with a decrease of \(\frac{\text{abs}_t}{\text{abs}_{700}}\) (photo-bleaching, at about 700 nm), which corresponds to an increase in the concentration of oxidized states of PSI reaction centre (P700\(^+\)) and plastoquinan (PC\(^+\)), accordingly, an MR/MR_{0} increase indicates P700\(^-\) and PC\(^+\) reduction.

The stored data are transferred to the computer and processed using a special software provided by the company, which provides plotting of PF, DF and MR kinetics, calculation of biophysical parameters according to the JIP-test [7-9], averaging data from repeated experiments, data transfer to other programs and numerical processing of data from DF kinetics in the dark including deconvolution of DF decay-curves into separated kinetic components (see Table A2).

### 2.3. The JIP-test

The JIP-test equations are based on the Theory of Energy Fluxes in Biomembranes [5]. Therefore, we chose here to demonstrate the definitions and equations of the JIP-test using the scheme of Fig. 1 (modified after [9]), which is the well-known Z-scheme of photosynthesis expressed by sequential energy fluxes (wide arrows), i.e., as energy cascade. Formulae and glossary of terms used by the JIP-test are presented in Table A1.

The energy cascade starts from absorption (ABS) by PSI antenna pigments and ends at the reduction of the end electron acceptors at the PSI electron acceptor side (RE) driven by PSI. Intermediate energy fluxes are the trapping flux (TR), defined as the energy flux leading to the reduction of plastoquinone (Pheo) and QA, and the electron transport flux (ET) that refers (see definitions) to the electron transport further than QA. At each of the steps, the energy influx is bifurcated to an outflux for energy conservation via electron transfer (grey arrows) and an outflux for dissipation (white arrows; note that TR-ET is the energy flux leading to the accumulation of QA\(_{\text{ox}}\)).

The efficiencies, as fractions of energy influxes that are transformed to energy outfluxes leading to energy conservation, are also indicated (next to the line arrows between sequential steps), where \(\varphi\) refers to quantum yields (efficiencies on absorption basis; i.e., fluxes per ABS), \(\psi\) to efficiencies per TR and \(\delta\) to efficiency per ET. For each energy bifurcation, the complementary of the respective efficiency (i.e., the fraction of energy influx that is transformed to energy outflux that does not lead to energy conservation via electron transfer: white arrow) is indicated in brackets under the corresponding outflux.

The scheme presents also the equations by which the quantum yields and the other efficiencies at the onset of illumination (all RCS open; subscript “O” are defined and further linked with fluorescence signals selected from the OJIP fluorescence transient, namely F\(_{0}\), F\(_{1}\), F\(_{2}\) and F\(_{3}\) (\(= F_{O}\)). The equations by which the quantum yields are linked with fluorescence signals are simple applications of the general equation of Paillotin [21]; according to this equation, the quantum yield at any time \(t\), where the fluorescence intensity is \(F_t\) (between \(F_2\) and \(F_3\)), is \(\varphi_{F_t} = 1 - F_t/F_3 = \Delta F_t/F_3\) (note: the equation \(\varphi_{F_1} = \Delta F_1/F_3\) is now known as the “Genty equation”, after being re-introduced in [22]). The Paillotin general further links (as shown) the quantum yield at any time \(t\) with the maximum quantum yield and the complementary of the relative variable fluorescence \(V_t\) at that time, as \(\varphi_{F_t} = \varphi_{F_1} (1 - V_t)\). The formula by which \(V_t\) is defined on the basis of fluorescence signals, \(V_t = (F_1 - F_0)/(F_M - F_0)\), is given at the bottom of the figure, along with the formulae defining the total electron carriers per reaction centre (ECo/RC), the specific energy fluxes (energy fluxes per RC; arbitrary units) and the performance indexes P_{ABS} and P_{Total}.

Note: In this scheme, RC refers to the active (QA\(_{\text{red}}\); reducing) PSI reaction centre.

### 3. Results

#### 3.1. Multi-signal information obtained with the M-PEA

\(H. \text{rhodopensis}\) leaves, attached on the plants, were measured with M-PEA after dark adaptation for 1 h, as described in the Material and methods section. Fig. 2 presents the multi-signal information obtained by illuminating a leaf-sample with a 30 s pulse of strong red actinic light (627 nm peak; 5000 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)). The kinetics (induction curves) of prompt and delayed fluorescence (PF and DF, in different a.u.; left vertical axis) and modulated 820 nm reflection (MR; right vertical axis) are plotted on logarithmic time scale from 20 ms to 30 s; this time, which is the analytical time for the PF and MR kinetics, is denoted as “JIP-time” in order to distinguish it from the analytical “delay-time” during which DF is recorded (see Material and methods).

The prompt fluorescence transient has the typical shape with the polyphasic rise OJIP and the subsequent decline towards the light-adapted state [4]; the steps are marked with open circles but, for clarity reasons, only O and P are labelled. The fast rise is generally accepted to reflect the accumulation of the reduced form of the primary electron quinone acceptor QA, otherwise the closure of photosystem (PS) II reaction centres (RCs), which is the net result of QA reduction due to PSII activity and QA\(_{\text{red}}\) reoxidation due to PSI activity. When the photosynthetic sample is kept in the dark, QA is practically fully oxidized (hence all RCS are open) and the prompt fluorescence yield is minimal; due to the high time resolution of the instrument, the PF intensity registered at the onset of illumination (at the O-step; 20 \(\mu\)s) can well be considered as emitted with the minimal yield and, hence, denoted as F\(_{0}\). The maximum intensity F\(_{P}\) (at the peak P) depends on the achieved redox state of QA and acquires its maximum possible value (F\(_{M}\)); if the illumination is strong enough (above 500 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) red light) to cause the closure of all RCS [8], provided that all RCS can get closed, i.e., all are active in QA\(_{\text{red}}\) reducing [23] and the samples are dark-adapted [24].

The modulated reflection signals are presented in Fig. 2 by the MR/MR\(_{0}\) ratio, where MR\(_{0}\) is the value at the onset of the actinic illumination (taken at 0.7 ms, the first reliable MR measurement); changes of this ratio express changes of the oxidation state of P700 and PC (see Material and methods). Therefore, the MR/MR\(_{0}\) transient in Fig. 2 exhibits the accumulation of P700\(^+\) and PC\(^+\) (MR/MR\(_{0}\) decrease – fast phase) lasting until about 10 ms, and the subsequent net re-reduction of both by the intersystem electron carriers (MR/MR\(_{0}\) increase – slow phase) that levels off at about the same time as the PF transient (at about 500 ms). The end of the fast phase (minimal MR/MR\(_{0}\)) is a transitory steady state, with equal oxidation and re-reduction rates. The time during which MR/MR\(_{0}\) is minimal (about 7–30 ms) is in the range of the J-I phase of PF and the slow phase develops mainly in the range of the I-P phase of PF.

The DF induction curves (DF vs. JIP-time) presented in Fig. 2 were constructed from the kinetics of DF vs. delay-time recorded during the dark interruptions of the actinic light (see Material and methods and Table A2). Each of them plots the DF intensity (a.u.) at a certain delay-time point vs. the JIP-time at which the dark interval started; hence, a vertical line cutting the DF induction curves at any JIP-time expresses (in one dimension) the kinetics of DF vs. delay-time in the dark interval that starts at the corresponding JIP-time. Here, only a selection
of constructed DF curves is presented (for clarity reasons); for the number of data points in each delay-time kinetics and, concomitantly, the total number of constructed curves, see Table A2.

As shown in Fig. 2, the curve of DF measured at 0.02 ms delay-time (DF_{0.02 ms}; red closed points) consists of a fast rise to a peak I_1 (at 7 ms) and a subsequent polyphasic decline through a shoulder I_2 (at about 100 ms) and a long lasting plateau (between 0.5 and 10 s) where I_3 level is located (at 1 s). I_1, I_2, and I_3, marked with open red circles, are denoted according to Goltsev’s nomenclature [11,25]. The amplitude of the DF induction curves decreases with increasing delay-time, with the extent of the decrease being bigger at the I_1 than at the I_2 level and that at the plateau I_3 being the smallest; thus, at the latest delay-time, I_1 and I_2 levels are slightly above I_3 (see also Fig. 3A). We observe that, in all DF induction curves: (a) I_1 appears at the time where the rate of PF increase from the J-step towards the I-step is the highest and where, also, the fast phase (decrease) of MR/MR_0 ends; (b) I_2 appears in the range of the I-P phase of the PF; (c) the DF plateau corresponds to the PF plateau; (d) the last phases (declines) of DF and PF appear during almost the same time range.

Fig. 3A presents the kinetics of DF (in arbitrary units) vs. delay-time at the characteristic levels I_1, I_2, and I_3; as shown, these are DF decay kinetics. In the main plot, the DF decay kinetics are presented on logarithmic time scale, from 0.02 to 0.2 ms and from 0.01 to 0.9 ms (the common range for all three DF decay kinetics; see Table A2); the insert, where the natural logarithm of DF is presented on linear time scale from 0.02 to 0.2 ms (symbols as in the main plot), shows that each of the three curves consists of more than one exponential decays with different rate constants (otherwise, it would have been a straight line), as known from the literature [11]. Table A2 shows fitting equations that can be used for their deconvolution into a sum of exponential decays (this type of data processing was not in the scope of the present work). The I_2' and I_3' curves in the inset (open triangles and diamonds) are drawn by shifting the I_2 and I_3 curves respectively, so that they start (at 0.02 ms) from the same value as the I_1 curve. In this way, the three curves can be compared concerning both their actual and their normalized values; the latter facilitates the comparison of their shape. As demonstrated by Fig. 3A (main plot and insert), the three kinetics differ, even in the microsecond range, concerning their average decay rate, which is highest for the I_1 and lowest for I_3, meaning that faster components dominate at I_1 than at I_2 and I_3.

Since DF originates from the repopulated excited PSII antenna chlorophylls, it is emitted with the same fluorescence yield as PF. This means that the DF intensity measured at any JIP-time (and plotted in Figs. 2 and 3A) is proportional to the product of the rate of repopulation of excited PSII antenna Chls (resulting from back electron transfer, charge recombination in PSII RC and migration of excitation to antennae) and the fluorescence yield (PF/absorption) of the PSII antenna Chls at that time. We therefore calculated, for each JIP-time, the DF/PF ratio, which expresses the rate (in arbitrary units) of repopulation per absorption at that JIP-time. The kinetics of DF/PF vs. delay-time are presented in Fig. 3B, in the same way as the DF decay kinetics in Fig. 3A. Obviously, division of DF by PF does not affect the comparison of the average decay rates of the three curves, but only the relation among their amplitudes since PF is higher at I_2-time than at I_1-time and even higher at I_3-time. However, Fig. 3B was here included as it provides, exclusively, information for the repopulation rate.

Fig. 2. Kinetics (induction curves) of prompt and delayed fluorescence (PF and DF, in different a.u.; left vertical axis) and modulated 820 nm reflection (MR; right vertical axis) induced by a 30 s pulse of strong red actinic light (627 nm peak, 5000 μmol photons m⁻² s⁻¹) in H. rhodopensis leaves attached on the plants and dark-adapted for 1 h, measured simultaneously with M-PEA (Multifunctional Plant Efficiency Analyser; Hansatech Instruments) and plotted on logarithmic time scale from 20 μs to 30 s (JIP-time). The modulated reflection signals are expressed by the MR/MR_0 ratio, where MR_0 is the value at the onset of the actinic illumination (taken at 0.7 ms, the first reliable MR measurement). The DF induction curves (DF vs. JIP-time) were constructed from the kinetics of DF vs. delay-time recorded during the dark interruptions of the actinic light (see Material and methods and Table A2); each of them plots the DF intensity (a.u.) at a certain delay-time-point (indicated by the colour/symbol code) vs. the JIP-time at which the dark interval started. Hence, a vertical line cutting the DF induction curves at any JIP-time (like the three black dashed lines in the plot) expresses (in one dimension) the kinetics of DF vs. delay-time in the dark interval that starts at the corresponding JIP-time. Characteristic points of the DF vs. JIP-time curves, i.e., the peak I_1 (at 7 ms), the shoulder I_2 (at 100 ms) and I_3 (taken at 1 s, in the plateau) are marked with open circles on the DF_{0.02 ms} (DF at 0.02 ms delay-time point) curve and labelled. Open circles were also used to mark the O, J, I and P steps of the PF kinetics (for their labelling, see Fig. 4).
variable fluorescence is not eliminated, even after a DT of 34 h that results in RWC decrease down to 1%, but the I-step does not appear as distinct step at RWC below 15% (DT>13 h). The described F0 and Fp changes cannot be attributed only to changes of the corresponding fluorescence yields, since absorption of the excited cross section undergoes also changes upon drying, due to changes of chloroplast orientation, turgo, sample geometry and Chl content. Concerning the latter, we found that it did not change throughout the experiment (data not shown), as also previously reported [18,26].

The OJIP parts of the PF transients depicted in Fig. 4 were analysed by the JIP-test (see Fig. 1, Table A1 and Materials and methods). Fig. 5 presents the derived parameters, after they were normalized using as reference the corresponding values at 100% RWC. In the left panel selected parameters referring to the PSI photochemical capacity are presented vs. the RWC for all the measured water-content-states; the general observation is that, for RWC below 10% (extreme desiccation, indicated by the shaded area) all parameters exhibit pronounced decrease.

The formula for the maximum quantum yield of primary photochemistry, \( \varphi_{\text{PSII}} = (F_{\text{M}} - F_0)/F_{\text{M}} \), is valid for samples where all RCs are active in \( Q_\text{A} \) reducing and, hence, under strong actinic illumination they are closed at the P-level (\( F_\text{p} = F_0 \)). However, different stresses can cause transformation of RCs to “heat sinks”, where the excitation energy is dissipated as heat instead of being transformed to photo-chemical energy; hence the fluorescence yield of such units is the same as that of open RCs [8,29]. Concomitantly, \( F_\text{p} \) is lower than \( F_\text{M} \) and the calculated \( \varphi_{\text{PSII}} \) expresses the average of the true \( \varphi_{\text{PSII}} \) of active PSII units and the \( \varphi_{\text{PSII}} = 0 \) of the inactive; we come to the same conclusion on the basis of the biophysical definition \( \varphi_{\text{PSII}} = \frac{\text{TR}0/\text{ABS}}{\text{TR}0/\text{RC}} \), where \( \text{TR}0 \) is the trapping flux in active units (by definition, \( \text{TR}0 = 0 \) in the inactive), while ABS is the total absorption flux in all units [23].

The JIP-test analysis involves the calculation (in a.u.) of the trapping flux per active reaction centre (\( \text{TR}0/\text{RC} \) and, hence, the calculation of RC/ABS = \( \frac{\text{TR}0/\text{ABS}}{\text{TR}0/\text{RC}} \); note that, in the JIP-test, the abbreviation RC is used only for the active PSII reaction centres (see Fig. 1 and Table A1; for reviews, see [7,8]). This permits us to distinguish whether an observed decrease, upon a treatment, of the calculated \( \varphi_{\text{PSII}} \) is due only to an increase of the nonphotochemical de-excitation rate constant \( k_\text{q} \) (quenching at the antenna) or to inactivation of a fraction of the reaction centres or to both. The three parameters mentioned above are presented in the left panels of Fig. 5, together with the \( k_\text{p}/k_\text{q} \) ratio (where \( k_\text{p} \) the photochemical de-excitation rate constant). The \( k_\text{p}/k_\text{q} \) ratio is equal to the \( F_\text{p}/F_\text{q} \) ratio (where \( F_\text{p} = F_\text{M} - F_0 \); maximum variable fluorescence); though it does not bring additional information (note that \( \varphi_{\text{PSII}} = k_\text{p}/(k_\text{p} + k_\text{q}) \), i.e., \( \varphi_{\text{PSII}} \) is determined by the same rate constants), it is a more sensitive parameter and provides a further criterion for the changes taking place in PSI structure, as follows: Since \( k_\text{p} \) corresponds to the average from active (true \( k_\text{p} \)) and inactive \( (k_\text{q} = 0) \) PSI units, the \( k_\text{p}/k_\text{q} \) ratio would be proportional to the RC/ABS if no change in \( k_\text{q} \) would occur [23] and diverge from the proportionality if \( k_\text{q} \) would also change.

Fig. 5 (left panel) shows that the decrease of the RWC results in inactivation of reaction centres. In the 100–30% RWC the decrease of active RCs is more pronounced than that of \( \varphi_{\text{PSII}} \) \( \equiv \text{TR}0/\text{ABS} \) and, accordingly, \( \text{TR}0/\text{RC} \) increases in this range (up to 20% of the initial value). In the 30–10% RWC range, no further inactivation occurs and the \( \text{TR}0/\text{ABS} \) decrease follows that of \( \text{TR}0/\text{RC} \). The increase, in the 100–30% RWC range, of \( \text{TR}0/\text{RC} \) (the only functional parameter in this plot; the other three are structural parameters) indicates that the absorption by the active units increases; further investigation is needed to explain this finding. The comparison of \( k_\text{p}/k_\text{q} \) with RC/ABS shows that \( k_\text{q} \) also increases (deviation of the \( k_\text{p}/k_\text{q} \) curve from the RC/ABS curve) when RWC decreases; the increase becomes significant for RWC below 30%.

The right panel of Fig. 5 depicts the efficiencies (structural parameters) for the whole energy cascade – from absorption to reduction of end electron acceptors at the PSI acceptor side – and the performance

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**Fig. 3.** Panel A: Kinetics of delayed fluorescence DF (in arbitrary units) vs. delay-time (DF decay kinetics) at the characteristic steps 1, 2, and 3 (at 7, 100 and 1000 ms JIP-time, respectively), indicated by closed circles, triangles and diamonds, respectively. The kinetics correspond to the thin dashed vertical lines in Fig. 2. Panel B: Delay-time kinetics of the ratio of DF to prompt fluorescence PF at the same JIP-times (1, 2, and 3, as indicated); the DF/PF ratio at each step expresses the rate (in arbitrary units) of repopulation of excited Chl at that step. The kinetics are plotted on logarithmic time scale, from 0.01 to 0.9 ms (the common range for all three DF decay kinetics; see Table A2). In the inserts of panels A and B, the natural logarithm of DF and DF/PF, respectively, is presented on linear time scale from 0.02 to 0.2 ms (symbols as in the main plots). The I2  and I3 curves (open triangles and diamonds) are drawn by shifting the I1 and I2 curves respectively, so that they start (at 0.02 ms) from the same value as the I1 curve. In this way, the three curves can be compared concerning their actual values (closed points) and their normalized values (closed points for I1 and open for I2 and I3), where the latter facilitates the comparison of their shape.

3.2. Analysis of the multi-signal information for investigating the response of *H. rhodopensis* to water loss

The multi-signal measurements were applied to screen dark-adapted leaves of *H. rhodopensis* being at different water-content-states, established by their gradual drying and after re-watering, aiming to investigate the strategy employed by the photosynthetic machinery of this plant that belongs to the few desiccation tolerant (resurrection) higher plants. The results are presented separately for each type of signal obtained.

Prompt fluorescence transients of *H. rhodopensis* leaves are depicted in Fig. 4. The transients, measured and induced as described for Fig. 2, are plotted on logarithmic time scale from 20 μs to 30 s (JIP-time). The sequence of the transients from the top to the bottom of the plot follows the decreasing relative water content (RWC) of the leaves (increasing duration of drying-time, DT), as indicated.

The earliest effect is a lowering of the P-level (\( F_\text{p} \)), which decreases progressively with increasing drying-time (DT), while \( F_\text{M} \) exhibits a slight increase (maximal extent 15% of the value at 100% RWC). The

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**Table A1:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F_\text{p} )</td>
<td>Prompt fluorescence</td>
</tr>
<tr>
<td>( F_\text{M} )</td>
<td>Maximum fluorescence</td>
</tr>
<tr>
<td>( \varphi_{\text{PSII}} )</td>
<td>Maximum quantum yield of primary photochemistry</td>
</tr>
<tr>
<td>( k_\text{p} )</td>
<td>Photochemical de-excitation rate constant</td>
</tr>
<tr>
<td>( k_\text{q} )</td>
<td>Nonphotochemical de-excitation rate constant</td>
</tr>
<tr>
<td>RC/ABS</td>
<td>Absorption by the active units</td>
</tr>
<tr>
<td>TR0/RC</td>
<td>Trapping flux per active reaction centre</td>
</tr>
<tr>
<td>TR0/ABS</td>
<td>Total absorption flux</td>
</tr>
</tbody>
</table>

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**Fig. 5:** Left panel: shows that the decrease of the RWC results in inactivation of reaction centres. In the 100–30% RWC the decrease of active RCs is more pronounced than that of \( \varphi_{\text{PSII}} \) \( \equiv \text{TR}0/\text{ABS} \) and, accordingly, \( \text{TR}0/\text{RC} \) increases in this range (up to 20% of the initial value). In the 30–10% RWC range, no further inactivation occurs and the \( \text{TR}0/\text{ABS} \) decrease follows that of \( \text{TR}0/\text{RC} \). The increase, in the 100–30% RWC range, of \( \text{TR}0/\text{RC} \) (the only functional parameter in this plot; the other three are structural parameters) indicates that the absorption by the active units increases; further investigation is needed to explain this finding. The comparison of \( k_\text{p}/k_\text{q} \) with RC/ABS shows that \( k_\text{q} \) also increases (deviation of the \( k_\text{p}/k_\text{q} \) curve from the RC/ABS curve) when RWC decreases; the increase becomes significant for RWC below 30%.

The right panel of Fig. 5 depicts the efficiencies (structural parameters) for the whole energy cascade – from absorption to reduction of end electron acceptors at the PSI acceptor side – and the performance
Fig. 4. Prompt fluorescence transients of dark-adapted H. rhodopensis leaves that were at different water-content-states established in darkness (for clarity reasons only a selection of measured transients is presented). The transients, measured with M-PEA (as in Fig. 2), were induced by red actinic light of 5000 μmol photons m⁻² s⁻¹ and plotted on logarithmic time scale from 20 μs to 30 s (JIP-time); the steps O (at 20 μs), J (at 2 ms), I (at 30 ms) and P (peak) are marked. The sequence of the transients from the top to the bottom of the plot (closed and open circles alternatively) follows the decreasing relative water content (RWC) of the leaves that is indicated (in %) for each curve, followed, in brackets, by the duration (in h) of drying-time (DT). The RWC vs. DT is depicted in the insert; the line was drawn using all measured water-content-states, while the points correspond to the selected transients presented in the main figure (closed and open circles, accordingly).

Fig. 5. Parameters quantifying the structure of the photosynthetic machinery of dark-adapted (1 h) H. rhodopensis leaves that were at different water-content-states established in darkness. The parameters (for their definition, see Fig. 1 and Table A1), derived by the JIP-test from the fast rise (OJIP) transients of the prompt fluorescence (see Fig. 4 and legend), were normalized using as reference the corresponding values at 100% RWC. In the left panels, selected parameters referring to the PSII structure are presented vs. the RWC, for all the measured water-content-states. The shaded area indicates the range in which RWC decrease results in a pronounced decrease of PSII photochemical capacity down to inhibition. The right panel depicts the efficiencies for the whole energy cascade – from absorption to the reduction of end electron acceptors at the PSI acceptor side – and the performance indexes, for selected water-content-states (indicated in the panel together with the drying-time).
indexes (potentials governed by the structural parameters), for selected water-content-states (not in the range of extreme desiccation). We observe that all presented parameters are affected by the RWC decrease, though at a different extent.

Fig. 6 refers to the delayed fluorescence (DF$_{20\mu s}$) signals (DF at 20 $\mu$s after the interruption of the actinic illumination – 20 $\mu$s delay-time) of dark-adapted H. rhodopensis leaves that were at different water-content-states established in darkness, obtained with M-PEA (as in Fig. 2) during the analytical JIP-time. In panel A, DF$_{20\mu s}$ induction curves, measured simultaneously with the PF transients of Fig. 4, are plotted on logarithmic time scale from 0.3 ms to 30 s JIP-time. In panel B, DF$_{20\mu s}$ at selected JIP-time points (corresponding to the dashed vertical lines in panel A) are plotted vs. the RWC of the leaves; the selected JIP-time points correspond to I$_{1}$ (7 ms), I$_{2}$ (100 ms) and I$_{3}$ (1 s) levels (see Fig. 2), including also the 30 ms JIP-time, a point between I$_{1}$ and I$_{2}$. We observe that, upon RWC decrease, the DF$_{20\mu s}$ transient decreases in amplitude and changes in shape, with the effect being more pronounced at the I$_{1}$ level, less at the I$_{2}$ and even less at the I$_{3}$ level.

In Fig. 6, panels C and D present the (DF$_{20\mu s}$/PF)$_{JIP}$-time-t, in the same way as panels A and B present the DF$_{20\mu s}$. As explained above (for Fig. 3B), DF/PF expresses the rate of repopulation of excited Chl per absorption (in arbitrary units). We observe that the shapes of (DF$_{20\mu s}$/PF)$_{JIP}$-time-t at selected JIP-time points (corresponding to the dashed vertical lines in panels A and C) vs. the RWC of the leaves.
PF)_{\text{JIP-time}}$ vs. JIP-time (panel C) are different than those of DF_{20\mu s} vs. JIP-time (panel A). To facilitate the comparison, we plotted in the insert of panel C the DF and DF/PF induction curves (DF standing for DF_{20\mu s}) at 100% RWC, normalized at the I1 level; we see that in the DF/PF curve the shoulder I2 is less pronounced than in the DF curve (see also Fig. 3), while the decline after the plateau is not exhibited at all. Upon RWC decrease, the (DF_{20\mu s}/PF) transient decreases in amplitude and changes in shape (panel C), with the effect being more pronounced at the I1 level and less at the I2 level, while the I3 level remains almost unaffected (panel D). At I2 the effect of drying on the DF/PF (panel D) is less extended than on DF (panel A) and, moreover, the shoulder at I2 disappears with the RWC decrease.

Decay kinetics of the repopulation rate, expressed by DF/PF vs. delay-time (as in Fig. 3B), at the characteristic steps I1 (main plot) and I2 and I3 (inserts), hence denoted as (DF/PF)$_{I_{1,2,3}}$, are presented in Fig. 7, after being normalized on (DF$_{0.02\text{ ms}}$/PF)$_{I_{1,2,3}}$, the corresponding value at 0.02 ms delay-time. (Note: [DF$_{I_{1,2,3}}$/PF$_{I_{1,2,3}}$] = [(DF)$_{I_{1,2,3}}$/[(DF)$_{0.02\text{ ms}}$/PF$_{I_{1,2,3}}$]]. Since the normalization cancels PF; however, we preferred to keep the expression DF/PF because of its meaning). The kinetics, obtained at the same water-content-states as the PF transients presented in Fig. 4 and the DF induction curves in Fig. 6 (A and C), are plotted on logarithmic time scale, from 0.02 to 0.9 ms. Closed circles were used for the kinetics obtained at states established after a drying-time DT ≤ 15 h (RWC ≥ 11.6%) and open circles for kinetics obtained after longer DT (lower RWC), as indicated in the main plot. Fig. 7 clearly shows that, at all three levels I1, I2 and I3, the decrease of the average decay rate of DF/PF upon RWC decrease is much more pronounced for RWC below 12%.

The kinetics of the normalized modulated reflection at 820 nm (MR/MR$_0$) induced by red actinic light of 5000 μmol photons m$^{-2}$ s$^{-1}$ in dark-adapted H. rhodopensis leaves that were at different water-content-states established in darkness are depicted in panels A and B of Fig. 8. The kinetics were recorded, as in Fig. 2, simultaneously with PF and DF transients, a selection of which is presented in Figs. 4 and 6A respectively, and plotted on logarithmic time scale from 0.7 ms to 30 s (JIP-time). Each curve presents the average of kinetics recorded during the indicated DT (and RWC) range. The separation of the curves in two panels (A and B) facilitates the distinction of two different effects of RWC decrease. Panel A, corresponding to the RWC decrease from 100% down to 10–15%, shows that drying results in the decrease, down to elimination, of the slow phase that indicates the re-reduction of P700$^+$ and PC$. Panel B, corresponding to the further RWC decrease, from 10–15% down to 1%, shows the decrease, down to elimination, of the fast phase that indicates the oxidation of P700 and PC. The shape of the (MR/MR$_0$) kinetics was further translated to characteristic parameters and plotted vs. the corresponding RWC (bottom abscissa) and DT (top abscissa) in panels C and D (for all measured water-content-states). The shaded areas in panels C and D (corresponding to panel B) indicate the RWC range in which RWC decrease results in progressive limitation, down to inhibition, of light-induced P700 and PC oxidation.

Re-watering of haberlea leaves that were at the end of the drying treatment (1% RWC) was found to fully restore the 100% RWC. With the complete rehydration, all signals were recovered, i.e., the OJIP transient -both as amplitude and shape- hence all the JIP-test parameters, the DF decay kinetics (hence, also the DF induction curves) and the MR/MR$_0$ kinetics (data not shown).

### 4. Discussion

Many experimental techniques are available today for the investigation of the energetic behaviour of a photosynthetic system. There is a general agreement that at room temperature, Chl $\alpha$ prompt fluorescence (PF) of plants, algae and cyanobacteria, in the 680–740 nm spectral region, is emitted mainly by photosystem (PS) II and it can therefore serve as an intrinsic probe of the fate of its excitation energy [27]. Since this fate is determined by the structure and redox poise of all the components, from water splitting until the PSI electron acceptors, a suitable analysis of PF kinetics can provide a wealth of information, not only for PSII, but for the whole photosynthetic process.

Such an analysis is the JIP-test [6–9], which links the different steps and phases of the fast PF rise OJIP with the efficiencies of electron transfer in the intersystem chain and to the end electron acceptors at the PSI acceptor side (Fig. 1). However, as for any model that is proposed in bioenergetics, any PF analysis is based on dogmas and assumptions. The dogma adopted in the formulation of the JIP-test model, like by the majority of researchers, is based on the theory of Duyens and Sweeney [28] that the fluorescence yield of PSI antenna is maximal when the PSII primary quinone electron acceptor QA is reduced (QA$^-$) and minimal when QA is in the oxidized state; the RCs are denoted as closed and open and the fluorescence intensities as F$m$ and F$o$, respectively. Between these extremes there are several biochemical redox states, since there are several combinations of redox states of the intersystem electron carriers (especially of QA, QB and PQ); all of them can be predicted by modelling and approximated by numerical simulations. Different conceptual viewpoints have been proposed, which consider the dogma of having a mixture of open and closed RCs too simplistic and which can be defended today with the same experimental precision. This means that the question of how the photosynthetic sample really works remains open.

Models of any theoretical complexity level can be formulated, but they are meaningful only if they can be experimentally validated [23]. For several decades now the need of obtaining multi-signal information has been recognized and according instrumentation has been developed and used. However, new questions create the need of new instruments. As shown in the present work, the M-PEA, used here for the first time, responds to such a need; the simultaneous measurements of PF, DF and MR allow collecting and correlating complementary information for all three domains of the photosynthetic
electron transport – PSII electron donor side, electron transport between PSII and PSI, and PSI electron acceptor side.

The utilization of the multi-signal recordings in monitoring the response of the photosynthetic machinery in *H. rhodopensis* leaves during drying and upon rehydration aimed to advance the understanding of the desiccation tolerance strategies of *H. rhodopensis* and, by comparing the different water-content-states where the photosynthetic machinery is not destroyed but only inactivated (reversibly) at different degrees, to recognize the differences in respect to each of the three signals and their correlation, which would also help to test and advance the current interpretations of each signal. The analysis of the prompt fluorescence (PF) transient OJIP was taken as the guiding signal, since it has been thoroughly analysed for many years on the basis of a bioenergetic concept.

As shown in Fig. 2, the modulated reflection at 820 nm (MR) recorded simultaneously with PF and with the same data acquisition, exhibits a fast decrease, corresponding to the accumulation of P700+ and PC+ followed by a slow increase corresponding to the net re-reduction of both by the intersystem electron carriers (as also previously reported [10]). The end of the fast phase appears at the time (7 ms) when the rate of PF increase from the J-step towards the I-step is maximal and the time range during which MR/MR₀ remains minimal (transitory steady state) corresponds to the J–I phase of PF (Fig. 2). The J–I phase has been interpreted as reflecting the progressive reduction of the plastoquinone (PQ) pool [8,10]. The transitory steady state of MR/MR₀ kinetics observed in the range is in good agreement with this interpretation: the accumulation of PQH₂ initiates electron transfer to PC+ and P700+, compensating the further oxidation of PC and P700 by PSI activity. The reduction rate overcomes then the oxidation rate, thus leading to MR/MR₀ increase (slow phase), during the I–P phase of PF; this supports the postulation that the I-step reflects the kinetic bottleneck of the electron transport chain between PQH₂ and cytochrome (cyt) b₅₆₃ (note that the halftime of the PQH₂ reoxidation by cyt b₅₆₃/f is 20 ms) and the attribution of I–P phase to the filling up of the available electron acceptors of PSI (see [29] and references therein). We note that, in the JIP-test, the definition of the quantum yield RE₀/ABS and the efficiency RE₀/ET₀ (RE abbreviates the reduction of end electron acceptors at the PSI acceptor side) and the
The basic innovation of the M-PEA instrument is that delayed fluorescence is recorded simultaneously with PF and MR and with the same high data acquisition. It should also be noted that this is the first DF investigation where actinic light of so high intensity (5000 μmol photons m−2 s−1) was applied. So far, DF has been investigated by three methods (for a recent review, see [11]); (a) by recording and analysing the DF dark relaxation (decays) after illumination with a short saturating flash applied to dark-adapted samples (see, e.g., [30,31]); (b) using phosphoroscopic methods that create light-dark cycles, DF was recorded during a part of the dark phase (and PF during the light phase) and the DF emission was collected during the whole DF recording interval and averaged to construct a point in the DF induction curve (see, e.g., [32]); (c) also with phosphoroscopic methods, DF dark relaxation in 0.35–5.5 ms dark intervals was recorded (about 100 digital values) and analysed, with the first dark interruption after 11 ms actinic illumination [33–35]. It is therefore clear that M-PEA brings an advancement of the third method since PF, DF and MR are recorded simultaneously with one instrument and the kinetics of DF vs. delay-time (DF decay kinetics) are obtained with high time resolution in dark intervals lasting from 100 μs to tens of seconds and at different JIP-times, with the earliest dark interruption at 0.3 ms (JIP-time). Moreover, different actinic light intensities, up to 5000 μmol photons m−2 s−1, can be used. The DF signals collected in the present study are multidimensional, since they depend on the JIP-time at which the dark interval started, the delay-time (during the dark interval) to which they refer (both times being analytical times) and the experimental time, during the drying treatment of the leaves (drying-time DT), at which they were recorded.

The DF intensity undergoes wide changes during JIP-time, exhibiting several maxima and minima, which have been labelled by Goltsnev and co-workers as “I” and “D” respectively and numbered in sequence according to their position in the induction curve (I1, I2,..., D1, D2,...); from the comparison of DF induction curves reported in [11,33–36], it appears that the number and relative amplitudes of the maxima and minima are strongly affected by the actinic light intensity used. With the high intensity used in the present study (5000 μmol photons m−2 s−1), the DFmax induction curve exhibits only a peak at 7 ms (I1), a shoulder (local maximum) at 100 ms (I2) and a long lasting plateau in the time range that later peaks (I3, I4,...) appear when lower actinic light intensities were applied; in this plateau, the point at 1 s was taken here as the I3 step. As shown in Fig. 3A, the kinetics of DF vs. delay-time at I1, I2 and I3, starting from different intensities, decay with different rates, with that of I1 being the highest and that of I3 the lowest. The ln(DF) kinetics show that, even in the 0.2 ms time range presented (insert of Fig. 3A), each decay is composed of different decaying components (otherwise it would have been a straight line), with the contribution of the fast components dominating the decay at I1 and being less at I2 and even less at I3.

According to the knowledge of today (see [11] and references therein), the recorded DF decay kinetics at any JIP-time is composed of several components, each with a different lifetime and amplitude, emitted because of back electron transfer and charge recombination at several PSII redox states (denoted here as “light emitting states” - LESs), such as P680+ “Pheo”−, P680+ Q−A, Z−Q−A, S−Z−Q−A Qb, S−Z−Q−A Q−A (written in the sequence from shorter to longer lifetimes). These states are formed by PSI photochemical activity and by sequential electron transfer in the electron transport chain. For the different LESs, the overall rate constant of back electron transfer and charge recombination is different; moreover, it may be also affected by thylakoid membrane energization, to which P700+ accumulation also contributes [37–39]. Speaking about the back electron transfer and charge recombination that lead to DF emission, we should keep in mind that they also take place during illumination though, obviously, the resulting luminescence cannot be distinguished from PF. This is why, at the moment that illumination is interrupted and DF can be recorded, there is a mixture of states, each of which decays with a different rate. It is also known that the intensity of slower DF components is lower than that of the faster [11]; moreover, the LESs where the oxygen-evolving complex (OEC) is at the S1- or S2-state lead to much lower DF intensity [31]. Therefore, the changes of DF during JIP-time, as presented by the DF induction curve in Fig. 2, are related with changes of the relative contribution of the LESs, since their concentrations are governed by electron transport reactions in the donor and acceptor sides of PSII and by the state of the oxygen-evolving complex (see, e.g., [34,40–43] and, for more references, [11]). It should also be emphasised that the lifetime of an LES is determined not only by a back electron transfer that leads to charge recombination and DF emission, but also by a forward electron transfer from, or to, the one of the two separated changes (“leakage type” [44]). Obviously, the rate of forward transfer depends also on the redox state of the other electron carrier involved in this transfer; e.g., the decay of Z−Q−A is determined by the rate of charge recombination (to ZQ−A) and by the rate of reoxidation of Q−A, which decreases when Qb is reduced (Qb or Q−b) and PQH2 accumulates. This explains why DF decay (reflecting the decay of the responsible LESs) is slower at I3 and even slower at I1 than at I2 (Fig. 3A), since Q−A, Q−b and PQH2 accumulate after the J-step of PF and, concomitantly, why the DF induction curves in Fig. 2 that were constructed by DF signals at different delay-times during the dark intervals, differ among them concerning both their amplitude and their shape.

As analysed above, the rate of charge recombination is determined by the concentration of the different LESs and the overall rate constant of back electron transfer and charge recombination for each of them. On the other hand, DF depends both on the rate of repopulation of excited Chls (rate of charge recombination multiplied by the probability that the free energy liberated is transformed to excitation energy of the antenna Chls) and on the fluorescence yield of the antenna Chls, like PF depends both on the absorbed light flux (hence, the excitation influx) and the fluorescence yield. At any JIP-time, the fluorescence yield is determined by the fraction of open (or closed RCs), which ranges between zero and unity; in general, the fluorescence yield is also determined by the photochemical and nonphotochemical de-excitation rate constants of the antenna (kD and kN, respectively), but the duration of the OJIP rise (1 s) is too short to affect them [8]. We therefore calculated the DF/PF ratio, which expresses the rate of repopulation (since the fluorescence yield is cancelled in the ratio) and we plotted the delay-time kinetics (Fig. 3B) and the induction curves (Fig. 6C) of this ratio. As shown in the insert of Fig. 6C, the DF/PF induction curve at 100% RWC exhibits a distinct I1 step and an I2 shoulder (though

formulae with which they are linked with F1 and F2 (see Fig. 1; also Table A1) were based on this postulation [9].

The strongest support of the postulation concerning the I–P phase comes from the comparison of the OJIP and the MR/MR0 transients, obtained during the drying of H. rhodopensis leaves (Figs. 4 and 8A and B, respectively). We observe that with the increase of the drying-time (DT), the initiation of both the I–P rise of PF and the slow phase of MR/MR0 are progressively shifted to longer JIP-times and their amplitudes progressively decrease; below 15% RWC (DT>13 h), the I–P disappears as a distinct step in the PF rise and the MR/MR0 slow phase does not develop at all. It is thus concluded that, while the MR/MR0 transient under normal conditions, exhibiting both the fast and the slow phase (Fig. 2 and the trace at 100–70% RWC in Fig. 8A), expresses the achievement of balance in the non-cyclic electron flow through both photosystems, the disappearance of the MR/MR0 slow phase (trace at 15–10% RWC in Figs. 8A and B) reveals that the two photosystems are disconnected (after PQH2). Concerning the PF signal, the two situations are indicated by a normal OJIP shape and an OJP shape respectively (Fig. 4). Similar findings were reported for pea leaves treated with dibromothymoquinone (DBMIB), which binds to cyt b6/f and inhibits the electron flow from PQH2 to PC [29]. The advantage of the present work is that the inhibition was not induced by an external reagent but by internal regulations, naturally employed, and which are, moreover, reversible (as found after rehydration of haberlea leaves; data not shown); in addition, we could follow here the progressive disconnection of the two photosystems.

The advantage of the present work is that the inhibition was not induced by an external reagent but by internal regulations, naturally employed, and which are, moreover, reversible (as found after rehydration of haberlea leaves; data not shown); in addition, we could follow here the progressive disconnection of the two photosystems.
less pronounced than in the DF induction curve), while the last decline (after the plateau where \( I_2 \) is located) does not appear at all; the latter shows that the decline in the DF curve (Fig. 6A) is solely due to the decrease of the fluorescence yield (Fig. 4).

If we compare, for the state with 100% RWC, the induction curves (JIP-time kinetics) of \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) (Fig. 6C) and PF (Fig. 4) we observe that they both increase until the \( I_1 \) step (beginning of J-P phase) but, after this step, PF further increases while \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) decreases. In order to explain the parallel and anti-parallel phases, we start from the postulation that the \( \mu \) and sub-ms components of DF originate from \( S_{\text{Z}}^{-}\text{Q}_{b}^{+}\text{Q}_{a} \), which is the most luminescent LES [34]. Therefore, \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) is determined by the concentration of this LES at the moment that the illumination is interrupted, which, in turn, is determined by the rate of RCs closure (Qa reduction) just before the illumination was interrupted; as explained above, the LESs with \( \text{Q}_{b}^{+}, \text{Q}_{a}^{-} \) and \( \text{PQH}_{2} \) that are subsequently formed, are less luminescent and have smaller decay rate constants. In other words, \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) depends on the trapping flux just before illumination was interrupted. Concomitantly, \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) is expected to decrease with the decrease of open RCs, i.e., during the development of the PF rise. This is indeed observed after the \( I_1 \) step, but cannot explain the parallel rises before \( I_1 \), for which we propose the following: After dark adaptation, OEC is mainly in the \( S_1 \) state and, therefore, the low luminescence state \( S_{\text{Z}}^{-}\text{Q}_{b}^{+}\text{Q}_{a} \) is formed before it is transformed to the high luminescence state \( S_{\text{Z}}^{0}\text{Q}_{b}^{+}\text{Q}_{a} \) to complete this transformation, every RC needs to absorb two quanta. Under further illumination, the S-states get unsynchronized (approximately equally distributed) and there are no S-state net transitions that would affect DF intensity [11]. So, the \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) increase until \( I_1 \) reflects the formation of \( S_{\text{Z}}^{0}\text{Q}_{b}^{+}\text{Q}_{a} \) due to \( S_1 \) to \( S_2 \) transformation, while the subsequent decrease reflects the decrease of this LES because of the decrease of open RCs that can close. This is supported by the observation that \( I_2 \) appears at the time where the rate of PF increase after the J-step is the highest (and where the fast phase of MR/MP ends, as above discussed). Moreover, as we observe by comparing Fig. 6C with Fig. 4, this holds true for all water-content-states. Extending this explanation, we can also relate the \( I_3 \) shoulder (where the less luminescent \( S_{\text{Z}}^{-}\text{Q}_{b}^{+}\text{Q}_{a} \) and \( S_{\text{Z}}^{0}\text{Q}_{b}^{+}\text{Q}_{a} \) predominate) with the PF rise after the \( I_1 \)-step and point out that this shoulder and the \( I_3 \)-step disappear at the same water-content-state.

In this frame we can now attribute the decrease of the amplitude of the DF/PF curve caused by RWC decrease (Fig. 6C-D) to the decrease of active RCs, which is in agreement with the results obtained by the JIP-test analysis of PF and shown in the left top panel of Fig. 4. The increase of \( k_{N} \) revealed in the latter cannot be responsible for the decrease of the DF/PF amplitude, since changes of \( k_{N} \) affect the fluorescence yield, which is cancelled when DF is divided by PF. The RWC decrease was also found to result in slower DF decays (Fig. 7) and, equivalently, to increase the lifetimes of the responsible LESs. We postulate that this is due to the decrease of the forward electron transfer because of the progressive disconnection of the two photosystems and the concomitant enhancement of \( \text{Q}_{b}, \text{Q}_{a}^{-} \) and \( \text{PQH}_{2} \) accumulation. This enhancement may also enhance back electron transfer; we speculate that the concomitant increase of DF, superimposed on the general decrease related with RCs inactivation, can explain why, upon RWC decrease, \( \text{DF}_{0.02\text{ ms}}/\text{PF} \) undergoes less extended decrease at \( I_3 \) than at \( I_1 \) and remains unaffected at \( I_3 \) (Fig. 6D).

In our discussion so far, the information derived from the simultaneously recorded PF and DF signals has been proven to be very efficient tools for studying the bioenergetics of native samples (see the reviews [5] and [11] respectively and references therein). Their simultaneous recording – including also the MR signal – with one instrument does not simply facilitate the collection of information. As shown in the present work, the main advantages, related also with the high time resolution of M-PEA, are the collection of a wealth of information, much more than by the separate recordings so far, and the correlation of complementary information for the whole photosynthetic electron transport, from \( \text{H}_{2}\text{O} \) to the end electron acceptors of PSI, all these with a light pulse of one to several seconds duration.

For the full exploitation of the large amount of obtainable information by M-PEA, construction of conceptual and mathematical models is necessary to link together all three signals. When the models are fitted to the experimental curves, we expect to obtain the values of rate constants of different photosynthetic reactions, which will further increase the applicability of the method in plant biology and agriculture.

Acknowledgements

R.J.S. and S.Q. acknowledge support by China 863 Program (2006AA1002A214) and 111 Program (B07030). R.J.S and M.T.-M. acknowledge support by the Swiss National Science Foundation, Project No. 200021-116765; V.G. thanks the Bulgarian National Science Fund, Project No. DO 02-137/15.12.2008 for the financial support, Liljana Maslenkova for providing the haberelea plants and Maria Gurmanova for experimental assistance.
Appendix A

Table A1
Glossary, definition of terms and formulae of the JIP-test parameters (see also Fig. 1) used for the analysis of the chlorophyll fluorescence transient OJIP emitted by dark-adapted photosynthetic samples (modified after [10]); fluorescence (F) refers only to prompt fluorescence and RC to the active (Qa reducing) PSI reaction centres.

<table>
<thead>
<tr>
<th>Data extracted from the recorded fluorescence transient OJIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F_i )</td>
</tr>
<tr>
<td>( F_{300} )</td>
</tr>
<tr>
<td>( F_{500} )</td>
</tr>
<tr>
<td>( F_t \equiv F_{300} )</td>
</tr>
<tr>
<td>( F_r )</td>
</tr>
<tr>
<td>( t_{ex} )</td>
</tr>
<tr>
<td>Area</td>
</tr>
</tbody>
</table>

Fluorescence parameters derived from the extracted data

| \( F_0 \equiv F_{300} \) | Minimal fluorescence, when all RCs are open |
| \( F_M \equiv F_0 \) | Maximal fluorescence, when all RCs are closed (= \( F_0 \) when the actinic light intensity is above 500 \( \text{mmol} \text{ photons m}^{-2} \text{ s}^{-1} \) and provided that all RCs are active as \( Q_a \) reducing) |
| \( F_t \equiv F_{300} - F_0 \) | Variable fluorescence at time \( t \) |
| \( F_r \equiv F_{300} - F_M \) | Maximal variable fluorescence |
| \( S_{300} \equiv \text{Area}(F_{300} - F_0) = \text{Area}(F_0) \) | Normalized area |
| \( V_t \equiv \text{Area}(F_t - F_0) / (F_{300} - F_0) \) | Relative variable fluorescence at time \( t \) |
| \( M_0 \equiv (\Delta F / \Delta t)_0 / (F_{300} - F_{300}) \) | Approximated initial slope (in ms\(^{-1}\)) of the fluorescence transient normalized on the maximal variable fluorescence \( F_v \) |

Fluorescence parameters derived from the fluorescence parameters

| EC/RC = \( S_{300} / \text{Area}(F_{M} - F_0) \) | A measure of total electron carriers per RC |

De-excitation rate constants of PSII antenna

| \( k_N \equiv \text{(ABS)} k_C (1 / F_M) \) | Nonphotochemical de-excitation rate constant (ABS: absorbed energy flux; \( k_C \): rate constant for fluorescence emission) |
| \( k_P \equiv \text{(ABS)} k_C (1 / F_0 - 1 / F_M) = k_P (F_0 / F_M) \) | Photochemical de-excitation rate constant |

Biophysical parameters derived from the fluorescence parameters

| EC/RC = \( S_{300} / \text{Area}(F_{M} - F_0) \) | A measure of total electron carriers per RC |

Table A2
Protocol for prompt fluorescence (PF), modulated reflection at 820 nm (MR) and delayed fluorescence (DF) measurements with M-PEA, during a JIP-time from 10 ms to 300 s. For each JIP-time digitalization range (1–7), the PF and MR data acquisition and the number of registered data points are tabulated, as well as the number and duration of dark intervals (d.i.) and the number of DF data points registered in each d.i. (with data acquisition according to the digitalization range(s) in the d.i.). The fitting equations for the DF decays [11] are also shown.

<table>
<thead>
<tr>
<th>JIP-time digitalization range</th>
<th>PF and MR</th>
<th>DF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recorded every</td>
<td>Number of d.i.</td>
<td>Duration of d.i.</td>
</tr>
<tr>
<td>(1) 0–300 ( \mu )s</td>
<td>10 s</td>
<td>10 s</td>
</tr>
<tr>
<td>(2) 0.3–3 ms</td>
<td>100 s</td>
<td>10 s</td>
</tr>
<tr>
<td>(3) 3–30 s</td>
<td>1 s</td>
<td>27</td>
</tr>
<tr>
<td>(4) 0.03–0.3 s</td>
<td>10 s</td>
<td>27</td>
</tr>
<tr>
<td>(5) 0.03–3 s</td>
<td>100 s</td>
<td>27</td>
</tr>
<tr>
<td>(6) 3–30 s</td>
<td>10 s</td>
<td>27</td>
</tr>
<tr>
<td>(7) 30–300 s</td>
<td>10 s</td>
<td>27</td>
</tr>
</tbody>
</table>

Fitting equations of DF decays

| | \( L_1 \) e\(^{-\tau_1} \) + \( L_2 \) |
| | \( L_1 \) e\(^{-\tau_2} \) + \( L_2 \) e\(^{-\tau_1} \) + \( L_3 \) |
| | \( L_1 \) e\(^{-\tau_4} \) + \( L_2 \) e\(^{-\tau_7} \) + \( L_3 \) e\(^{-\tau_2} \) + \( L_4 \) |
| | \( L_1 \) e\(^{-\tau_4} \) + \( L_2 \) e\(^{-\tau_7} \) + \( L_3 \) e\(^{-\tau_2} \) + \( L_4 \) e\(^{-\tau_5} \) + \( L_5 \) |
| | \( L_1 \) e\(^{-\tau_4} \) + \( L_2 \) e\(^{-\tau_7} \) + \( L_3 \) e\(^{-\tau_2} \) + \( L_4 \) e\(^{-\tau_5} \) + \( L_5 \) |
| | \( L_1 \) e\(^{-\tau_4} \) + \( L_2 \) e\(^{-\tau_7} \) + \( L_3 \) e\(^{-\tau_2} \) + \( L_4 \) e\(^{-\tau_5} \) + \( L_5 \) |

Subscript “0” (or “o”) when written after another subscript indicates that the parameter refers to the onset of illumination, when all RCs are assumed to be open.
References


