1. Introduction

In oxygenic photosynthesis the two photosystems (PSII and PSI) operate in series during the transport of electrons from H₂O to CO₂. This implies a serious constraint on the antenna systems serving these photosystems, since turnover by the two photosystems must be equal in the white light that the photosynthetic system is adapted to utilize (more details in Discussion). This would not be a problem if the quantum efficiency of electron transport and pigment composition of the antennae were equal in both photosystems. Recent advances in molecular analysis of the photosynthetic antenna systems have revealed the numbers of Chls bound with different pigment–protein complexes (Table 1). For example, assuming a typical PSII dimer composition of C₅₅S₂M₂ (two cores, two strongly and two medium bound LHC) and taking monomer density (½C₅₅S₂M₂) of 1.2 μmol m⁻² and PSI density of 1 μmol m⁻², then there are 173 μmol Chl m⁻² associated with monomeric PSI and 175 with monomeric PSII, resulting in the ratio of excitation partitioning between PSII and PSI, q₂/q₁ = 1.01. In case of an extremely large PSII antenna, including two loosely bound LHCII per dimer (C₅₅S₂M₂L₂) and the same center densities 1.2 and 1 μmol m⁻², then there are 173 μmol Chl m⁻² associated with PSI and 226 with PSI, resulting in q₂/q₁ = 1.3.

Based on these analytically measured antenna sizes per PSII and PSI monomer, excitation partitioning between the photosystems is proportional to the ratio of the densities of photosystems. The latter generally indicates the dominance of PSI, varying from 2.5/1 to 1.2/1 depending on growth light quality in pea [1,2]. Based on excitation spectra of low-temperature PSI fluorescence, excitation partitioning to PSI was estimated to be only 0.3 in bean leaves over the visible range, with peaks approaching 0.5 at some wavelengths [3], indicating significant over-excitation of PSI in relation to PSI. Recently the excitation balance of the two photosystems was explored in relation to the quantum yield of CO₂ fixation in cucumber leaves grown under the sunlight spectrum or under shade light (preferentially exciting PSI) or blue light (preferentially exciting PSI) [17]. The relative excitation capture rates of the two photosystems, PSII/(PSI + PSII), calculated in vivo from Chl fluorescence and leaf transmittance signals at 810 nm, were strongly proportional to
the same ratio calculated from in vitro antenna size and photosystem density values, but the proportionality constant was about 1.3 in favor of the in vitro data.

If there are problems with balancing the two photosystems, the condition should be reflected in the action spectrum of photosynthesis [18]. The first report of unbalanced stimulation of PSI and PSII was the Emerson enhancement effect, when addition of short wavelength (PSII) light synergistically enhanced the quantum yield of photosynthesis under far red (PSI) light [19]. Subsequent measurements revealed a characteristic drop in photosynthetic quantum yield at 450–500 nm [20–22], suggested to be caused by over-excitation of PSI relative to PSII due to absorption by Chl b [23]. Instead of the physical spillover [24], in plants the partitioning of excitation is regulated toward an optimum by the “state transition” mechanism, which is the ability to redistribute LHCCI between PSII and PSI depending on excitation spectral composition [2,25,26]. Thus, though the total numbers of Chls are closely similar per photosystem and the most striking difference between PSII and PSI is in terms of the Chl a/b ratio and carotenoid content, in leaves the number of trimeric LHCCI may be variable dependent on growth conditions, leaving the actual antenna sizes of PSII and PSI, as well as the excitation partitioning ratio, open.

In this work we revisit the problem of photosystem excitation balancing, making use of recent advances in optical and O2 evolution measurements on leaves. We report the action spectra of PSI and PSII separately in terms of the global yield (with respect to all absorbed quanta) and analyze the intrinsic quantum yields (with respect to quanta exciting the particular photosystem) and excitation partitioning between the photosystems. In the blue part of the spectrum both photosystems are screened by pigments whose spectrum is similar to that of carotenoids. In the center of the sphere there were an object and reference holder with a 1000 W FEL type etalon lamp, model 63350 serial No. 7-1074, equipped with selected interference filters (when the absolute quantum yield of PSII was measured).

The quantum yield of PSII was measured with non-saturating single-turnover flashes (STF) generated by the Xe source via interference filters (10 nm band-width at half-height, Thorlabs, Newton, NJ). Quantum energy input, either integrated over time for flashes (µmol photons m−2) or as quantum flux rate of filtered white LED light (monochromatic, µmol photons m−2 s−1), was measured by a Miniature Fiber Optic Spectrophotometer PC 2000 (Ocean Optics, Dunedin, FL), spectrally calibrated against a 1000 W FEL type etalon lamp, model 63350 serial No. 7-1074, according to manufacturer’s instructions. Photon absorption by the leaf during a flash (µmol m−2) was calculated by multiplication of the filtered flash emission spectrum for leaf absorbance spectrum. The actual spectrum of the “monochromatic light” was calculated as the product of the emission spectrum of the light source and the transmission spectrum of each filter. At some wavelengths this significantly shifted the effective midpoint of the band.

2.2. Leaf chamber and illumination

A laboratory-made two-channel leaf gas exchange measurement system (Fast-Est Instruments, Tartu, Estonia) enabled control of CO2, H2O and O2 pressures and measurement of O2 evolution and transpiration. The leaf was enclosed in a 32-mm diameter by 3-mm deep chamber and flushed with gas at a flow rate of 0.5 mmol s−1. To stabilize leaf temperature and immobilize the leaf for optical measurements, the upper epidermis was sealed with starch paste to a glass window in contact with a water jacket. Gas exchange occurred through the lower epidermis. The leaf temperature was always within 0.2 °C of the water jacket temperature (22 °C).

The leaf chamber was illuminated via a fiberoptic light guide. Plastic fibers (1 mm, Toray Polymeric Fiber, PF series, from Laser Components, Gröbenzell/München, Germany) were individually arranged to produce uniform illumination of the chamber-enclosed adaxial leaf surface from three superimposed light sources. In this work one branch was used for FR illumination (LED 720–66–16100, Roithner Lasertechnik GmbH) and the second branch for additional green light (OD-520 L, Opto Diode Corp.). The third branch was connected to a STF source (Machine Vision Strobe MVS-7020, EG&G Optoelectronics, Salem, MA) or, alternatively, to a white LED (Enfis UNO Array 5 × 5 neutral white 4000–4500 K) equipped with selected interference filters (when the absolute quantum yield of PSII was measured).

2.3. Measurement of light absorption in the leaf

Leaf absorptance was measured with the PC 2000 using a leaf disk placed in an integrating sphere and illuminated by white light from a halogen source. The integrating sphere was made of compressed white Teflon powder. In the center of the sphere there was an object and reference holder side by side—a white horizontal metal sheet with two holes of 13 mm diameter. Light was guided into the sphere from the bottom by a single fiber, arranged so that most of the light cone passed through the object hole. Two pairs of recordings were made for one measurement. The fraction of stray light not illuminating the sample was measured, first, placing a black object on the sample holder and leaving the neighboring reference holder empty (reading R1) and, second, leaving the sample holder empty and placing the black body on the reference holder (R2). The fraction of stray light, Tblack = R1 / R2, was about 0.04. With leaf samples, first, a 15 mm leaf disk was placed on the sample holder and another, 15 mm white Teflon disk on the reference holder (R1), and then the places of the leaf and Teflon disk were exchanged (R2). The corresponding leaf scattering signal Tleaf = R2 / R1. Leaf absorptance (A) was calculated as

$$A = \frac{1 - T_{\text{leaf}}}{1 - T_{\text{black}}}$$ (1)
Along with photosynthetically active pigments, a small part of radiation was absorbed in cell walls and other non-pigment structures. We assumed that at 800 nm all absorption was non-photosynthetic and spectrally constant. Therefore, absorptance in photosynthetic pigments \((A_P)\) was found as

\[
A_P = A \cdot \left[ 1 - \ln \left( \frac{1 - A_{\text{800}}}{1 - A} \right) \right].
\]  

where parameters \(A\) and \(T\) in Eqs. (1) and (2) are wavelength dependent over the spectrum.

2.4. Measurement of PSI fluorescence

Non-variable fluorescence of PSI, typically 0.37\(F_0\) in the long-wave spectral window of 750 ± 20 nm, was calculated as the invariable part of \(F_0\) fluorescence present in the long-wave window, but absent in the short-wave window of 680 ± 10 nm. In overnight dark-adapted leaves fluorescence was measured in the two wavelength windows in the \(F_0\) and \(F_m\) states. The 750 nm signal was plotted vs. the 680 nm signal, a straight line was drawn through the two measured points and extrapolated to zero of the 680 nm signal. The obtained offset at 750 nm was considered to be PSI fluorescence [48,49].

2.5. \(O_2\) evolution measurements

Oxygen evolution was measured in the flow-through gas exchange measurement system with a calcium-stabilized zirconium \(O_2\) analyzer (S-3A, Ametek, Pittsburgh, PA, USA) on a background of 80 ppm \(O_2\) in \(N_2\) and 200 ppm \(CO_2\). The analyzer was calibrated against the atmospheric \(O_2\) concentration. The calibration has been confirmed by the ratio of \(O_2/CO_2\) fluxes of 1.0055 during photosynthesis in high light [28]. Due to the small volume of the leaf chamber and tubing, the half-response time of the system was only 0.6 s [29]. For steady-state measurements the reference (zero) was recorded after darkening the leaf. Respiratory \(O_2\) consumption was strongly suppressed due to the low ambient \(O_2\) concentration [30]. Density of PSI centers was measured as \(4 \cdot \delta_{O_2}\) evolution generated by an individual saturating STF [57].

2.6. 810 nm transmittance measurements

Leaf transmittance at 810 nm was measured with a laboratory-made modulated spectrophotometer [31]. A 810-nm LED (Type ELD 810-525, Roithner Lasertechnique, Vienna, Austria), filtered by a 40-nm band-pass interference filter (FB 800-40, ThorLabs), is driven by rectangular pulses of 5.5 μs at 90 kHz by a quartz-stabilized generator. The beam is applied to a 2-cm\(^2\) sub-area of the leaf surface. A fiber bundle collects transmitted radiation from the abaxial side of the leaf and guides it to a sensor PIN diode (S3590-01, Hamamatsu, Japan). An FB 800-40 band-pass interference filter (ThorLabs) minimizes the sensitivity to Chl fluorescence and non-modulated radiation. The PIN diode is operated under a constant counter-voltage of 10 V minimizing and stabilizing the internal capacitance of the diode. The photocurrent is amplified by a feedback controlled current-to-voltage converter, rectified, offset against 2 V, and the difference is further amplified.

2.7. Calculation of the redox state of P700

In these calculations the 810-nm signal was scaled to be 0 at complete oxidation (saturation pulse) and 1.0 at complete reduction (dark). The value of the 810-nm signal during pre-illumination was defined \(s\). From the condition of equilibrium [31,32]

\[
\frac{P_{700}}{P_{PC}} = k_7 \frac{PC}{PC^+}.
\]  

where the reduced fractions \(P700\) and \(PC\) were assumed to generate the positive optical signal. A quadratic equation describes the redox ratio \(r = P700 / PC^+\):

\[
r = \frac{-B + \sqrt{B^2 - 4AC}}{2A},
\]

where

\[
A = 1 - s; B = \frac{k_7}{\epsilon + \rho} + \frac{\rho}{\epsilon + \rho} - s(1 + k_7); C = -sk_7.
\]

and \(s = 4\), which is the ratio of extinction coefficients of \(P700\) and \(PC\) at 810 nm. \(p\) is the ratio of \(PC/P700\) in leaves (typically 2) determined from the oxidative titration of PSI donors by FRL [32,33] and \(k_7 = 30\) is the \(P700/PC\) equilibrium constant [31,32]. The redox state of \(P700\) is

\[
\frac{P_{700}}{P_{700}^+} = \frac{r}{1 + r},
\]

and the number of donor side electrons, per PSI, is

\[
n_{d} = \frac{r}{1 + r} + \frac{\rho p}{r + k_7}.
\]

2.8. Membrane isolation, pigment–protein complexes purification and pigment analysis

Stacked thylakoids were isolated from sunflower leaves as previously described [34]. Membranes corresponding to 400 μg Chl were washed with 5 mM EDTA and then dissolved in 800 μl of 0.7% α-DM, 10 mM Hepes, pH 7.5. Dissolved samples were then fractionated by ultracentrifugation in a 0.1 to 1 M sucrose gradient containing 0.06% α-DM and 10 mM Hepes, pH 7.5 (22 h at 280,000 g, 4 °C). Green bands corresponding to monomeric Lhcb, trimeric LHClIL, PSI core and PSI + LHCl complexes [35] were harvested. Absorption spectra were obtained in 10 mM Hepes, pH 7.5, 0.06% α-DM, and 0.2 M sucrose; measurements were performed using an SLM-Aminco DW-2000 spectrophotometer at room temperature. Pigments were extracted from complexes with 85% acetone buffered with Na2CO3, and then the supernatant of each sample was recovered after centrifugation (15 min at 13,000 g, 4 °C).

3. Results

3.1. General strategy

We seek to determine the intrinsic quantum yields (electrons transported per photon absorbed by the particular photosystem), \(Y_{II}\) and \(Y_{I}\) of PSI and PSI, respectively, and likewise the excitation partitioning coefficients \(a_0\) and \(q_0\) between PSII and PSI in leaves. To achieve the goals, we apply recent advances in studies of the photosynthetic electron transport in leaves—fast-response measurements of \(O_2\) evolution for PSII responses and precise 810 nm transmittance measurements for PSI responses. The spectrum of the global quantum yield of PSI, \(Y_{II}\) (electrons transported per all quanta absorbed by the leaf) was determined by measuring the \(O_2\) evolution rate under rate-limiting intensities of different wavelengths of monochromatic light. The global yield of PSI, \(Y_{II}\) was determined by comparison of the responses of PSI (\(O_2\) evolution) and PSI (the corresponding 810 nm transmittance response) to low-intensity flash illumination. The intrinsic yields of PSI (\(Y_{II}\) and PSI (\(Y_{III}\), electrons transported per photon absorbed by the photosystem) were determined by solving a system of equations, based mainly on the fact that in far-red light the intrinsic quantum yield of PSI, \(Y_{II}\) is close to the global yield \(Y_{II}\), as most photons are absorbed by PSI at these wavelengths (corrections were considered by measurements at two wavelengths). The obtained very high yield \(Y_{II} = 0.88\) leads to a conclusion that a small amount of antenna Chl is
needed to support the measured global yield \( Y_{II} \), while the larger part of Chls are joined with PSI. Despite of it, the measured global yield \( Y_{II} \) was still relatively low, revealing the intrinsic quantum yield of PSI, \( Y_{II} = 0.63 \), which is significantly lower than the yield of charge separation of 0.89, detected from Chl fluorescence.

3.2. Action spectrum of PSII from \( O_2 \) evolution

The global quantum yield of PSII oxygen evolution, \( Y_{II} \), was measured using weak monochromatic light. The leaf was pre-illuminated in far-red light (FRL, 706 nm, 198 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), 200 ppm\( \text{CO}_2 \) and 2\% \( \text{O}_2 \) concentration in \( \text{N}_2 \). Under this illumination PC was completely oxidized and P700 was only little reduced, as indicated by the 810 nm transmittance signal. PQ was also oxidized due to the strong over-excitation of PSI. For the measurement of quantum yield, first, the gas was changed to 80 ppm \( O_2 \) and the 200 ppm \( \text{CO}_2 \) and after 2 min the pre-illumination FRL was changed to a monochromatic light cut from the spectrum of a white LED by interference filters. The intensity of the light was chosen such that the rate of \( O_2 \) evolution did not change much compared to that under FRL (intensities of 10 to 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) dependent on wavelength). Fig. 1 shows a typical response of the \( O_2 \) evolution rate to a transition from the pre-illumination to the monochromatic measurement light. Oxygen evolution, initially supported by PSI excitation due to the pre-illumination light, rapidly changed dependent on the intensity of the monochromatic light (adjusted such that \( O_2 \) evolution only slightly increased at the wavelength of 462 nm in Fig. 1), stabilized for a second, and then linearly declined in the light with the rate that differed depending on excitation wavelength. It was checked that – with the baseline for \( O_2 \) measurements recorded in the dark – the rate of \( O_2 \) evolution was proportionally related to the intensity of the monochromatic light. Light-induced changes in respiratory \( O_2 \) uptake were minimized due to the very low background \( O_2 \) concentration, which strongly suppressed the respiratory \( O_2 \) uptake [30], and due to the fast-response measurements, eliminating slow readjustments in respiratory metabolite pools. Therefore, the global quantum yield \( Y_{II} \) was calculated as 4 times the initial (at 2.7 s, Fig. 1) \( O_2 \) evolution rate under the monochromatic light, divided by the monochromatic PAD. The later linear decline was caused by the accumulation of PQH\(_2\) due to over-excitation of PSI at this wavelength, as indicated by fluorescence rise (below). In terms of \( \text{e}^-/\text{per photon absorbed in the leaf, the global PSI yield, } Y_{II} \), was 0.42–0.43 in red and green light, but decreased to about 0.30 in the blue spectral range (Fig. 2). As anticipated, at wavelengths >680 nm the yield decreased rapidly.

Chlorophyll fluorescence was slightly above \( F_0 \) during pre-illumination, but decreased to the \( F_0 \) level when pre-illumination was turned off without the transition to the monochromatic light. When the pre-illumination light was changed to monochromatic light, fluorescence slowly and linearly increased in parallel with the decreasing \( O_2 \) evolution rate. The quantum yield of PSII charge separation, \((F_{m} - F) / (F_{m} - F_{o})\) was 0.89 in the beginning of the monochromatic illumination, considering PSI fluorescence as indicated in the figure. The gradual decline in \( O_2 \) evolution rate and increase in Chl fluorescence yield following the application of the monochromatic light indicated over-excitation of PSI in relation to PSI at this wavelength, leading to accumulation of PQH\(_2\). The relative difference in \( O_2 \) evolution rate, \( [(\text{initial rate} - \text{rate after } 3 \text{ s}) / \text{initial rate}] \), showed a complex action spectrum with maxima in the blue and red (Fig. 3). This result indicated that the two photosystems were not balanced indeed, but PSI was over-excited compared to PSI in blue and red light. We further investigated the quantitative extent of the misbalance in PSI and PSI excitation.

3.3. Optical flash-responses from PSI and PSII

Measurement of the global PSI yield, \( Y_{II} \), was more complicated than that of \( Y_{II} \), since no gas exchange signal could be directly related to activity of this photosystem. Information about PSI performance was extracted from the leaf transmittance change at 810 nm, which reports on the reduction state of P700 and PC, the primary and secondary electron donors to PSI.

In these experiments the leaf was pre-illuminated under combined green plus far-red light (GL, 540 nm, 18 \( \mu \text{mol m}^{-2} \text{s}^{-1} \times 0.72 \) absorption, plus FRL, 706 nm, 40 \( \mu \text{mol m}^{-2} \text{s}^{-1} \times 0.62 \)). Under these conditions PQ at the PSI acceptor side and PC at the PSI donor side were almost completely oxidized, but P700 was about half-reduced, being in redox-equilibrium with PC [31]. The pre-illumination was terminated and immediately followed by a weak monochromatic STF from the filtered Xe source.

The flash-induced 810-nm signal changes were small compared to the full amplitude of the 810-nm signal (Fig. 4). A correction for \( \text{Fd}^- \) signal, 15\% of the oxidation jump, was considered for the complete oxidation level, because \( \text{Fd}^- \) becomes reduced, decreasing the overall optical signal.
as soon as the donor side carriers PC and P700 become oxidized. Under the pre-illuminating GL + FRL light the steady-state equilibrium reduction of P700 was supported by linear electron flow from PSII excitation [36] and by the “dark re-reduction” of inter-photosystem carriers [37], which probably occurs via the same pathway as the fast, proton-uncoupled component of cyclic electron transport around PSI [33,38]. These electron transport pathways continued operation for some time in the dark after the background illumination was terminated, causing post-illumination reduction of P700+ and PC+, shown as the reference line measured by darkening the leaf without the flash (Fig. 4). In the exemplified recording a 650 nm weak STF was applied immediately after the background light was turned off. The difference between the measurement and reference traces represents the temporal response of the 810-nm signal to electron flow generated by the STF alone.

Fig. 5 illustrates the post-flash time course of this 810 nm difference signal in detail, an average over a large number of measurements. Clearly there is a delay of about 2 ms between the PSI flash-induced oxidation of the P700→PC equilibrium pair and the following PSI-induced reduction of these carriers, leaving sufficient time to separate the 810 nm optical responses from PSI and PSII electrons. The initial reduction state of P700 was between 0.4 and 0.5 in individual leaves, corresponding to a very small fraction of reduced PC (as illustrated in Fig. 4). As a result, the post-flash re-equilibration of the PC→P700 pair was fast and invisible in our recording, so the arriving PSII electrons were clearly distinguished from the PSI-induced oxidation of the donor side carriers. The statistics of electron transfer from flash-reduced PQ through Cyt b6f to PC→P700 rather exactly followed the Poisson (exponential) temporal kinetics with an average time constant of about 12 ms (but somewhat different in individual leaves).

3.4. Per electron response of the 810 nm signal and PSI density

For an individual leaf the (flash→reference) difference signals are shown in Fig. 6 for 650 and 713 nm wavelengths. The post-flash immediate response towards oxidation is the principal measure of the quantum yield of PSI at the flash wavelength, but for further analysis it had to be converted from % of the optical signal into electrons m⁻², in order to be compared with the amount of absorbed photons m⁻². The full amplitude of the exponential reducing phase was a good measure for the calibration of the optical signal, proportional with the amount of electrons generated by PSII during the flash (but overestimated by a small portion of the flash-transferred electrons cycling back from the acceptor side of PSI, see below).

Initially the PSI cycle was neglected and the 810-nm signal change was calibrated based on the known quantity of PSII electrons generated by the STF. The latter was calculated from the known amount of photons in the STF, multiplying the flash photon dose (assuming 7% loss due to double hits) by the global quantum yield of PSII (Fig. 2). This procedure resulted in a variable per electron signal (for brevity we use this term instead of μmol e⁻ m⁻²) dependent on flash wavelength (Fig. 7), indicating an evident correlation between the 810-nm per electron signal and leaf absorbance (apparent optical density). For an individual leaf the scattering of data was small and we obtained a strong non-linearly saturating dependence of the 810-nm per electron signal vs. leaf absorbance. The curve (Fig. 8) approached a saturating value at the absorbance of 0.6, the minimum in the green spectral area, but the data point measured at 713 nm – the extreme low values of absorbance and of the PSI yield – significantly jumped up from the smooth curve, indicating that the number of electrons arriving at the PSI donor side was underestimated. On this basis we assumed that not only PSII electrons caused the exponentially rising signal, but a fraction of electrons transferred to the PSI acceptor side by the flash cycled back to the donor side with temporal kinetics rather similar to those of the PSII electron transfer
through the Cyt b6f complex [38]. Inserting different values for the proportion of cycling electrons, the smoothest curve over the whole spectrum was obtained on an assumption that 15% of electrons, transferred to the PSI acceptor side by the flash, were cycled back to the donor side at all flash wavelengths (Fig. 8). The PSI cyclic flow had little influence on the results at wavelengths, where PSI produced many electrons per flash, but the influence was significant in far-red, where the contribution of PSI cyclic electron flow was about equal to the contribution of PSII electrons (Fig. 6).

The dependence of the 810 nm per electron signal on leaf optical density is evidently based on inhomogeneous flash excitation across the leaf interior. Blue and red photons were strongly absorbed in the leaf layers close to the upper surface. 810 nm photons sensing the oxidized P700 had a greater chance to escape from the leaf when the oxidized PSI centers were concentrated at the leaf surface. In green and far-red light the generated P700 was rather equally distributed oxidized PSI centers were concentrated at the leaf surface. In green oxidized P700 had a greater chance to escape from the leaf when the

gradient of P700°. Comparing the flash-oxidation signals with the Pm deflection we considered the different intra-leaf gradient by applying a normalization coefficient for the measured 810 nm deflection, extending from 1.0 for green and far-red light to 1.5 for blue light, as seen from the relative trend of the per electron signal in Fig. 8. Now, by comparison of the normalized 810 nm flash signal with the total 810 nm Pm signal we converted the relative optical signal to the relative number of electrons per PSI, involved in the flash oxidation—reduction procedure (Eq. (7)). As an example, we revealed that for the 650 nm flash, the amount of 0.199 μmol e− m−2 generated by PSI was equivalent to 0.186 electrons per PSI. This pair of figures allowed us to calculate the density of PSI, which was 0.199/0.186 = 1.07 μmol m−2. Thus, knowing the absolute number of flash-generated electrons m−2 by PSII on the one hand, and knowing the fraction of PSI these electrons reduced on the other hand, we obtained the density of PSI centers. Before the absorbance-dependent normalization of the 810 signal, the so obtained PSI density was variable dependent on wavelength of the flash, but remained constant after the signal was normalized to the most uniform intra-leaf distribution of P700°. Considering that in this leaf the PSI density was 1.76 μmol m−2 (measured as 4 · O2 evolution generated by saturating STF), the PSI/PSII ratio was 1.76/1.07 = 1.64.

3.5. Global quantum yield of PSI

For finding the global quantum yield of PSI, the relative number of electrons transferred per PSI was first multiplied by PSI density, μmol m−2, for finding the number of electrons transferred by PSI per m−2 of leaf. Further, the quantum efficiency of PSI electron transport was found by dividing the amount of transferred electrons by the dose of photons in the flash, μmol m−2. However, the so obtained figure characterizes the efficiency of those PSI, which had P700 reduced when the flash was applied. The global quantum yield of all PSI with their P700 reduced was found by dividing the above fractional yield by the fraction of PSI with reduced P700, calculated from Eq. (6) for the pre-conditioning light. The so calculated global quantum yield of PSI with reduced P700, Yf, is presented in Fig. 2 in comparison to the global quantum yield of PSII.

The result of Fig. 2 confirms the initial result of Fig. 3, indicating significant over-excitation of PSII in some spectral intervals. The global PSI yield, Yf, is generally lower than the PSII yield Yf, as shown in
wavelength and photosynthetically inactive pigments are absent. Defining the wavelengths as $\lambda_1$ and $\lambda_2$, we write

$$y_{\Pi} = \frac{Y_{I\Pi,1}}{1 - \frac{Y_{I\Pi,2}}{Y_{I\Pi,1}}} = \frac{Y_{I\Pi,1}}{1 - \frac{Y_{I\Pi,2}}{Y_{I\Pi,1}}}$$

(11)

yielding

$$y_{\Pi} = \frac{Y_{I\Pi,1} - Y_{I\Pi,2} - Y_{II,1}}{Y_{I\Pi,1} - Y_{II,2}}$$

(12)

and

$$y_{\Pi} = \frac{Y_{I\Pi,1} - Y_{I\Pi,2} - Y_{II,1}}{Y_{I\Pi,1} - Y_{II,2}}$$

(13)

These expressions contain only measured global quantum yields on the right side. One wavelength was chosen at 713 nm, where $Y_{\Pi} = 0.074$ and $Y_{I} = 0.776$ (Fig. 2). For better contrast the second wavelength was chosen at 648 nm, where PSII activity was high and the global yields were $Y_{I} = 0.435$ and $Y_{I} = 0.273$. Substituting these values into Eqs. (12) and (13) we obtained the intrinsic yields $y_{I} = 0.88$ and $y_{II} = 0.63$.

### 3.7. Partitioning of excitation between PSII and PSI

The above determined intrinsic yields allowed us to calculate the spectral excitation partitioning coefficients

$$a_{I}(\lambda) = \frac{Y_{I}(\lambda)}{Y_{I}}$$

and

$$a_{II}(\lambda) = \frac{Y_{II}(\lambda)}{Y_{II}}$$

(14, 15)

presented in Fig. 10. Characteristically, in the green and red spectral range PSII received about 0.65–0.68 but PSI only 0.32–0.35 of absorbed photons. A confirmation for our calculation routine came from the fact that applying Eq. (9) for different wavelengths the sum $a_{I} + a_{II}$ remained constant with $y_{I} = 0.88$ and $y_{II} = 0.63$ over the whole amber-red spectral range, where carotenoids did not absorb light.

In the blue spectral range the sum $a_{I} + a_{II}$ declined from unity, showing that about 30% of light was screened by photosynthetically inactive pigments, correspondingly reducing the availability of photons for photosynthesis.

For comparison with the in vivo kinetic data, pigment–protein complexes were isolated from a sunflower leaf and separated by sucrose density gradient as continuous curves. Black curve is the sum $a_{I} + a_{II}$; the two larger diamonds indicate data points used for calculation of $y_{I}$ and $y_{II}$ from Eqs. (12) and (13).
gradient ultracentrifugation as described in the Methods section. The absolute absorbance of pigments bound with PSII was significantly higher than the absorbance of pigments bound with PSI (Fig. 11). The fractional absorbance of the PSII core plus total Lhcb (denoted PSII + LHCII) was only a little higher than the functional in vivo excitation partitioning coefficient to PSI, at the red maximum of Chl absorption (Fig. 10). The fractional absorbance of PSI pigments was still rather significantly less than the calculated partitioning coefficient in the red part of the spectrum. A distinct difference between the absorbance of the pigment complexes and excitation partitioning to photosystems was evident at the short-wave end of the spectrum. At wavelengths < 580 nm the functional excitation partitioning to both PSII and PSI dropped by up to 30%, but this was not reflected in absorbance of the pigment complexes.

4. Discussion

In our hands the sum of the global quantum yields \( Y_{II} + Y_I \) was about 0.42 + 0.30 = 0.72 in the red part of the spectrum (Fig. 2), equivalent to 0.125 - 0.72 = 0.090 O₂ per photon absorbed by the leaf. This value is somewhat lower than 0.106, reported for net O₂ evolution of 37 C₃ species [39], but close to the yields of CO₂ uptake of 0.073 to 0.093 in C₃ plants under non-photorespiratory conditions [40–44]. We note that the measurements [39] were carried out with incandescent illumination deprived of the far-red part by short-pass filters, and measured with the LiCor quantum sensor. The latter has a very sharp cutting edge exactly at 700 nm, but most short-pass filters leave significant shoulder of transmittance from 700 to 720 nm, still active in photosynthesis [45]. As a result, in these measurements the photosynthetically active PAD was underestimated in the near far-red region, resulting in overestimated quantum yield (see also Fig. 12).

4.1. Charge recombination in PSI

Our kinetic analysis shows interesting spectral details, but generally it confirms the significant over-excitation of PSI compared to PSII in intact leaves, pre-illuminated with dominantly PSI light in our experiments. This raises the question of how electron transport is balanced for the two photosystems operating in series. Both photosystems are linked by linear electron flow, \( J_\text{ft} \), from H₂O to CO₂ and alternative acceptors (the latter being mainly N and O₂ [46]). Consequently, \( J_\text{ft} \) must be equal through both photosystems. In addition to the linear flow, both photosystems may operate with losses, first, due to excitation decay before charge separation, second, charge recombination (or electron cycling) after charge separation, such that their total excitation conversion rate is \( J_\text{f} = J_\text{ft} + J_\text{cin} \) and \( J_\text{f} = J_\text{f} + J_\text{cs} \), where the subscripts CII and CI indicate the decay/recombination/cycling rates in PSII and PSI. In this conceptual framework we focus on the low-light conditions used for the measurements of the quantum yield, where \( J_\text{f} \) involves physiological pathways of cyclic electron transport (ms time domain), recombination processes involving separated charges (ps time domain) and the \( F_\text{0} \) fluorescence-emitting process of excitation decay before charge separation (ps time domain), but no non-photochemical excitation quenching (NPQ).

In absence of losses the total quantum yield of the 2-photosystem mechanism could be \( Y_\text{f} = Y_\text{f} = 0.5 \) e⁻ per photon absorbed, but in our hands the yields were \( Y_\text{II} = 0.42 \) and \( Y_I = 0.30 \) in the red light (Fig. 2). Furthermore, our analysis succeeded in specifying that such a low global yield was mainly caused by significant losses of excitation energy in PSI, operating with an intrinsic efficiency \( \eta_\text{II} = 0.63 \) with respect to photons partitioned to this photosystem, while PSI operated with a higher yield \( \eta_I = 0.88 \). In our analysis \( \eta_I \) and \( \eta_\text{II} \) were found from a system of equations based on measurements at two wavelengths, one of which was chosen such where PSI activity dominated, and the other such where PSI activity dominated. Then the excitation partitioning coefficient \( \alpha_\text{II} \) was found such that it ensured the measured global yield \( Y_\text{II} \) and \( \alpha_\text{I} \) was found such that it ensured the measured global yield \( Y_I \). Since the system of equations was derived under an assumption that the sum \( \alpha_\text{II} + \alpha_\text{I} = 1 \), the latter condition was a priori fulfilled at the two wavelengths chosen for determination of \( \eta_I \) and \( \eta_\text{II} \). Correctness of the calculation method was confirmed by the fulfillment of the condition \( \alpha_\text{II} + \alpha_\text{I} = 1 \) at other wavelengths in the red part of the spectrum, where carotenoids did not absorb light (Fig. 10).

Intrinsic yields of the photosystems are of central importance for finding the excitation partitioning coefficients. The value of \( \eta_\text{II} = 0.88 \) is in good agreement with the value of 0.96 ± 0.11, determined on PSI trimer complexes from Synechocystis 6803 using the pulsed photoacoustic method [47]. In accordance with the high intrinsic yield, a relatively low excitation partitioning coefficient \( \alpha_\text{II} \) satisfies the measured global yield \( Y_\text{II} \). Consequently, most Chl is connected with PSI, in accordance with the PSII/PSI center density ratio of 1.64, obtained from our analysis.

It has been suggested that the excess of PSII centers over PSI centers is justified by the inevitable photoinhibition of PSI, as a result of which electron transport rates through both photosystems get balanced when about a half of PSII are inactivated [50]. In our experiments leaves were not exposed to high light intensities, eliminating the possibility of photoinhibition, thus the investigated state was an uninhibited natural
state of the photosynthetic machinery. At the low dominantly PSI light the quantum yield of charge separation in PSII was 0.89, as detected by the variable fluorescence \( F_v/F_m \) (considering \( F_m \) in the total \( F_v \)), but 29% of separated charges did not yield in \( O_2 \) evolution, resulting in the intrinsic yield of 0.63. The reason for the low yield, not accompanied by high fluorescence, could be charge recombination from an acceptor side carrier [51]. Such recombination did not occur from \( Q_a \), since it was not noticed in DCMU inhibited leaves [52], but rather charges recombined from a reduced form of \( Q_b \) [53]. This mechanism decreases the efficiency of PSI electron transport, but has no influence on the \( F_0 \) and \( F_m \) values reflecting charge separation, maintaining the \( F_0/F_m \) ratio constant.

Our finding of a low PSII yield in steady state at low light is in conflict with insignificant “misses” of excitation (< 10%) for the advancement of S-states [54]. Evidently, the low-yield state of PSI is not a principal property of this photosystem, but is adjusted in intact leaves during photosynthesis. The high yield of S-state advancement has been shown not for single photons, but mainly for saturating STFs (multiple photosynthesis. The high yield of S-state advancement has been shown not for single photons, but mainly for saturating STFs (multiple

contrast, the quanta of red light are absorbed by carotenoids and transferred to the chlorophylls, thereby enabling electron transport to continue. However, the efficiency of electron transport is lower when the light is predominantly red.

4.3. Spectrum of the quantum yields \( Y_{II}/Y_{I} \)

An important result of this work is the strongly conserved spectral distribution of the ratio of PSI/PSII quantum yields among broadleaf plant species (Fig. 9). If our optical measurements may seem unreliable, then Fig. 3 directly confirms that PSI activity was closely balanced with PSII activity only at wavelengths ~450, 500–530 and 630 nm, but PSI was overexcited at other wavelengths under our experimental conditions. The red maximum of PSI excitation at 646 nm is followed by the “red drop” at longer wavelengths. Other characteristic maxima in PSI excitation are located at 470–490 and rather widely between 540–590 nm. The minima of PSI excitation are in the extreme blue of 397–446 nm, plus two narrow bands, one at 518 and the other at 629 nm. As a result of this spectral imbalance the \( Y_{II}/Y_{I} \) ratio oscillates between 1.0 and 1.6 over the wavelength range from 400 to 680 nm.

Such a conserved spectral pattern of partitioning excitation between PSII and PSI confirms that the antenna systems are under strong genetic control, i.e., the number of the Lhc and Lhca units per photosystem core is rather constant, as are the relative abundances of the PSII and PSI super-complexes, though absolute numbers per leaf area unit may vary [66–69]. Moreover, the stoichiometry of PSI to PSI reaction centers in leaf segments from spinach, cucumber and tobacco, all grown in moderate light, was recently determined by two different approaches (electrochemical signal and electron paramagnetic resonance) and gave similar PSI/PSII ratio > 1 [50]. All these results are consistent with the constant Chl \( a/b \) ratio (around 3.2–3.5) measured in several plant species upon growth in full sun [70]. Considering also the data in Table 1 (Introduction), our results indicating a relatively large total PSI antenna (absorption cross-section) in leaves are consistent with the PSI/PSI ratio significantly exceeding unity – as obtained from our analysis – and/or dominating PSI composition of C252M212 in our leaves, as expected in State 1, where the loosely bound (L) LHClII trimer is attached to PSI.

Contrary to the visible part of the spectrum, where PSI is dominantly PSI light, facilitating the attachment of LHC to PSI (State 1, [25,26]). The movement of one LHClII from PSI in State 1 to PSI in State 2 [71] would shift the points of \( Y_{II}/Y_{I} \) vertically in Fig. 9, such that the maxima of over-excitation of PSI would decrease and PSI would become over-excited with respect to PSII at wavelengths where it is equally excited in State 1 [72]. It will be the object of further research to show how much state transitions can actually regulate the spectral \( Y_{II}/Y_{I} \) ratio in intact leaves.

4.4. Temporal kinetics of electron transport

Along with the quantum yields of both photosystems, these measurements produced also novel information about the temporal kinetics
of inter-photosystem electron transport in intact leaves. Using the 810-nm signal we recorded the movement of electrons from PSII to PSI after flash-excitation of the photosystems. During the initial 2 ms after the flash virtually no electrons passed through the inter-photosystem chain (Fig. 5). Subsequently, the number of electrons arriving at P700 increased mono-exponentially with a time constant of 12 ms at the leaf temperature of 22 °C (Fig. 6). The mono-exponential process characterizes the turnover of a single rate-limiting chemical reaction—most likely the Q-cycle of the Cyt b6f complex. It means that cytochromes b of the Cyt b6f complex were suitably pre-reduced during the preconditioning light, to ensure the transfer of both electrons from PQH2 to the donor side of PSI through the Q-cycle. Considering that our low-intensity flashes caused the release of a single PQH2 molecule from only 5% of PSI, the recorded P700 reduction rate represented the diffusional and Q-cycle processes involved in the processing of a single PQH2 molecule. With a single PQH2 molecule as substrate the Q-cycle rate of (12 ms) was only about a half slower than the theoretical maximum rate of electron transfer through Cyt b6f with maximum PQH2 concentration of about 6 PQH2 per PSI [73,74]. Thus, the kinetics of PQH2 oxidation by Cyt b6f are rather close to zero-order saturation at any realistic PQH2 concentration, beginning from 0.05 PQH2 per PSI. Our result also shows that in intact leaves the major rate-limiting step in inter-photosystem electron transport is not diffusion of plastiquinol, but its oxidation in the Q-cycle. Assuming that the initial sigmoidal part of the curve characterizes the release of the PQH2 molecule from PSI (1.6 ms, [29]), plus the diffusional delay, we see that diffusion of PQ from PSI to Cyt b6f is still much faster (2 — 1.6 = 0.4 ms) than the Q-cycle (12 ms). The short ~1 ms diffusion time and exponentially increasing probability of the PQH2 oxidation reaction in time do not reveal significant heterogeneities in inter-photosystem electron transport, e.g. such as percolation, resulting in domains with significantly different PQ/PSII ratio and largely variable diffusion time between the grana and stroma thylakoids [75–78].

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