

# Changes of Excitation Spectra of *in vivo* Chlorophyll Fluorescence during Induction of Photosynthesis

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Excitation spectra of chlorophyll fluorescence from intact rye leaves were registered at different steps of the induction of photosynthesis after dark adaptation. Analysis of these spectra indicates that at least two processes related to spectroscopic features are responsible for a fluorescence quenching. The first one, active during the first 100 s of illumination, was interpreted to consist in an overall decrease of the fluorescence quantum yield of antenna pigments and chlorophylls, in particular close to the reaction centers. The second type of a fluorescence decrease (between 100 s and 300 s of illumination) was found to be in large extent related to decrease of the rate of an excitation energy transfer between accessory xanthophyll pigments and chlorophylls emitting fluorescence. This latter molecular mechanism is discussed as being related to violaxanthin availability to de-epoxidation in the xanthophyll cycle.

## Introduction

Part of light captured by a photosynthetic apparatus is utilized to drive photochemical reactions. A portion of non-utilized absorbed energy is emitted back as a fluorescence. Analysis of a fluorescence originating from photosynthetic pigments *in vivo* was found to be a powerful tool in studying photophysical as well as photochemical aspects of photosynthesis (see refs. [1] and [2] for a review). An induction of photosynthesis is one of the processes which may be followed by means of fluorescence measurements. This possibility reported for the first time by Kautsky [3] has now the detail theoretical background based on photochemical and non-photochemical processes [1, 2] which may be studied separately by means of the modulated light technique [4]. Several recent studies seem to suggest that non-photochemical quenching of chlorophyll fluorescence in photosynthetic apparatus origins mainly from the light-harvesting pigment-protein complex of photosystem II (LHCII) [5–8]. According to the Horton's group such fluores-

cence quenching is related to acidification of LHCII environment [5, 9, 10] followed by its apoprotein aggregation [5–7]. On the other hand light-induced chlorophyll fluorescence decrease in isolated LHCII was demonstrated to be independent of the aggregation state of the antenna protein [8]. Fluorescence quenching of isolated LHCII was also found to be independent of the xanthophyll cycle-controlled violaxanthin to zeaxanthin ratio, the reversibility of this quenching being however much faster in the zeaxanthin presence (Gruszecki, Krupa, and Strasser, in preparation). Such a result corresponds well to the finding that zeaxanthin acts as an amplifier of fluorescence quenching, determined however in reversibility measurements [5, 10]. In the present study the process of a decrease of fluorescence during the induction of photosynthesis after dark adaptation is analyzed by means of a fluorescence spectroscopy from the point of view of participation of particular groups of photosynthetic pigments in this physiological process.

## Materials and Methods

Winter rye (*Secale cereale* L., cv. Pastar) was cultivated in a greenhouse with supplemented light and a relative humidity of 60%. Ten days-old rye

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leaves used for fluorescence measurements were dark adapted for 30 min directly before measurements. Whole intact leaves were placed in a spectrofluorometer with 1 cm fragment stretched on a quartz-glass slide (2 cm from the top of a leaf). There was right angle between excitation and emission beams (angle of incidence  $45^\circ$ , with respect to the flat sample). Before and after fluorescence measurements absorption spectra of a leaf were recorded with a Shimadzu 160A spectrophotometer and stored in memory. Fluorescence measurements were performed with a Shimadzu RF 500 spectrofluorometer equipped with a solid sample holder. Fluorescence excitation spectra were corrected with the use of Shimadzu spectra correction attachment. In order to avoid large deformation of registered spectra, related to photosynthesis induction, spectra were recorded in a fast mode (17 nm/s). Between subsequent measurements of excitation spectra leaf was illuminated with a probing light of spectrofluorometer (600 nm, 20 W/m<sup>2</sup>) for a certain period of time, indicated. All fluorescence measurements were done with following slit widths: excitation slit 5 nm, emission slit 10 nm. Simultaneous measurements of a time course of fluorescence changes were performed in a simultaneous measurements mode of apparatus.

Light-harvesting pigment-protein complex of photosystem II (LHCII) was isolated from ten days-old rye leaves. LHCII was isolated and purified by the method of successive cation precipitation as described in detail previously [11, 12]. Pure LHCII was suspended and examined in 50 mM Tricine-NaOH buffer (pH 7.8) containing 50% by volume glycerol. Fluorescence measurements of LHCII were done at total chlorophyll concentration of 0.9 mg/ml. Liquid sample was placed in a 0.3 mm width glass cell placed in the Shimadzu solid sample holder.

Violaxanthin was extracted from rye leaves with ethanol and purified by means of thin layer chromatography on silica gel plates with benzene:ethyl acetate:methanol solvent mixture (75:20:5, v:v:v) as a developing phase [13]. Isolated LHCII was modified with an exogenous violaxanthin at an endogenous to exogenous carotenoid ratio of 75:1. The total carotenoid concentration in LHCII preparation was determined to be as high as 470 µg/ml. In order to modify LHCII with vio-

laxanthin, preparation was placed in a glass tube having the thin film of a pigment deposited by evaporation in nitrogen. The suspension of pigment-protein was then sonicated three times for 10 s at room temperature. All measurements were performed at room temperature,  $25^\circ\text{C} \pm 1$ . Concentration of photosynthetic pigments in LHCII preparation was evaluated in ethanol according to the procedure of Lichtenthaler [14]. In order to determine violaxanthin to zeaxanthin ratio V/Z, ethanolic extracts from five different leaves after five independent experiments (in two series: five before and five after illumination) were mixed together and carotenoids were separated as described above for violaxanthin purification. Xanthophyll concentration was determined spectrophotometrically on the basis of extinction coefficients reported elsewhere [13]. Every kind of fluorescence measurements was repeated at least for 20 times and all spectroscopic features reported here were found to be highly reproducible.

## Results and Discussion

Fig. 1 presents the excitation spectra of *in vivo* chlorophyll fluorescence registered from dark-adapted rye leaf and after illumination of the same leaf for a period of 1 min and a subsequent 5 min. The difference spectra presented in the panel B of this figure show an absorption profile of the group of pigments responsible for a fluorescence decrease in the course of illumination of a photosynthetic apparatus during the first minute and between the sixth and the first minute. The two main maxima in the first spectrum: detected at 440 nm and around 470 nm could be assigned as corresponding to chlorophyll *a* and chlorophyll *b* Soret band, respectively. The intensity of 440 nm band compared to that at 470 nm is evidently higher in the case of the first difference spectra than in the original excitation spectra of the leaf at every time of illumination. This is an indication that the process of a fluorescence quenching at its very beginning is not simply related to the decrease in the quantum yield of fluorescence of all light-emitting pigments in the same pattern. The "peripheral" accessory pigments, in a terminology of a "funnel model" of photosynthetic light harvesting [15], are distinctly less involved in this process. The fluorescence decrease is probably connected with the emission

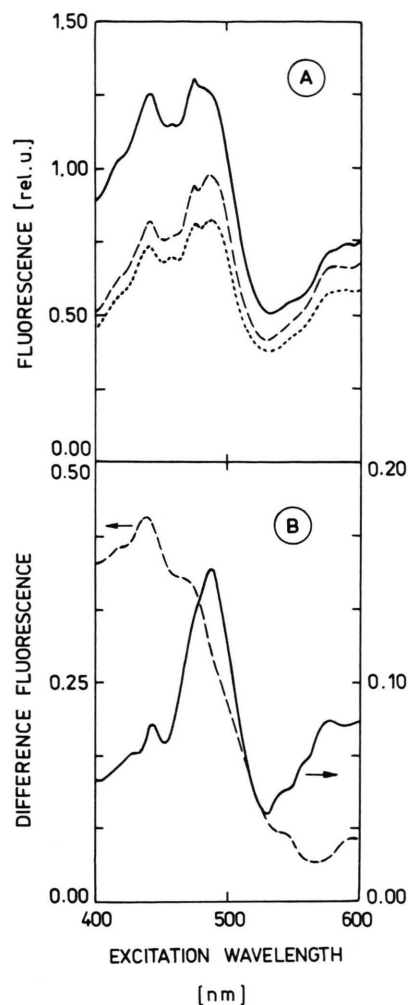


Fig. 1. (A) Fluorescence excitation spectra (emission at 680 nm) of an intact rye leaf, dark-adapted for 30 min (—) and, after subsequent illumination for 1 min (---) and following 5 min (····) with a probing light of spectrofluorometer (600 nm, 20 W/m<sup>2</sup>). (B) Difference spectra produced by subtracting spectra presented in panel A: the first one (—) minus the second one (---) gives the spectrum drawn with dashed line, the second one (---) minus the third one (····) gives spectrum drawn with solid line. For more experimental details see text.

coming from the reaction centers, regulated directly by photochemical processes [1, 2]. The second difference spectrum (solid line, Fig. 1 B) is dominated by a single band with a maximum around 490 nm. The same band was interpreted as represented xanthophylls acting as accessory pigments in the light-harvesting pigment-protein complex of photosystem II (LHC II) [16]. As can be seen from

the difference spectra (Fig. 1 B), this band is accompanied by an enhancement of fluorescence intensity in the spectral region between 560 and 580 nm. This specific and very reproducible feature might be interpreted as representing the electronic transition to the symmetry-forbidden first excited, singlet energy level  $^1A_g^*$  reported for the first time for carotenoid pigments by Thrash *et al.* [17]. This energy level was postulated to participate directly as an energy donor in the process of the singlet-singlet photosynthetic energy transfer between carotenoids and chlorophylls [18], considered as an antenna function. An existence of the band assigned to  $^1A_g \rightarrow ^1A_g^*$  transition in the same spectral region as in the present study was reported in the case of violaxanthin [19], known as an accessory xanthophyll pigment of LHC II [20]. The second mechanism of a fluorescence quenching during the induction of photosynthesis, represented by the second difference spectrum may be then summarized as an energetic uncoupling of a certain pool of carotenoid pigments from fluorescent chlorophylls. It is noteworthy that this mechanism was found in the present study as a response to a moderate actinic light, in physiological conditions. The similar mechanism of energetic "disconnection" of carotenoid pigments was reported previously for some stress conditions: senescence [21] and photoinhibition [22, 23]. This indicates for a sensitivity of a carotenoid-chlorophyll energetic coupling to different conditions and suggests the possibility of its regulatory function. In order to follow continuously the molecular mechanism referred to above as energetic uncoupling, the time courses of a fluorescence emission excited at 440 nm and 490 nm were recorded simultaneously (Fig. 2 A). Fig. 2 B presents the time dependence of the ratio of an intensity of fluorescence excited at these both wavelengths, Ex 440/Ex 490. During the first 40 s of illumination of a leaf the ratio Ex 440/Ex 490 followed the fluorescence changes: rapid rise and a subsequent decrease. In our opinion such a behavior is an indication that spectral forms of photosynthetic pigments excited at 440 nm (represented in numerator of the ratio) are mostly responsible for a fluorescence quenching at this stage. This is more evident in a subsequent phase (40 s till 100 s) where the ratio decreases with no dependency of the local fluorescence changes (see Fig. 2). Starting from  $t = 100$  s the

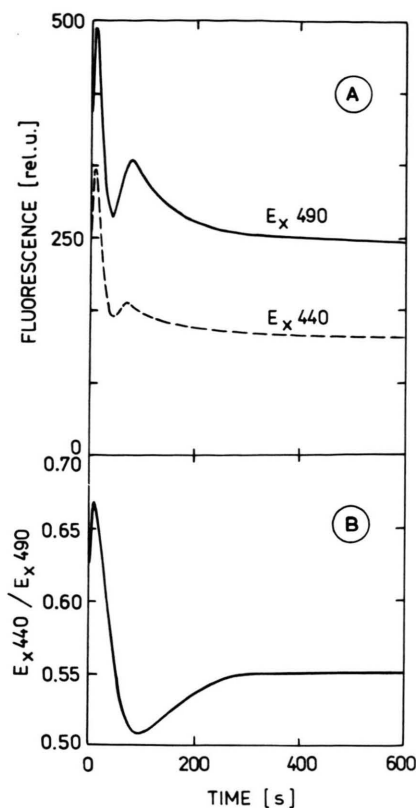


Fig. 2. (A) Simultaneously recorded time-dependence of fluorescence changes of an intact rye leaf excited at 440 nm and 490 nm (emission at 680 nm). (B) Time course of the ratio of a recorded fluorescence intensities presented in panel A:  $E_{x440}/E_{x490}$ . Leaf was dark-adapted for 30 min before the experiment.

$E_{x440}/E_{x490}$  increased and remained constant after  $t = 300$  s, approximately. These changes were accompanied by the gradual decrease of a fluorescence excited at both wavelengths. The observed increase in the ratio is an indication that spectroscopic forms of photosynthetic pigments active in this spectral region, namely xanthophylls, are primarily responsible for a decrease in a rate of emitted quanta. The same conclusion was drawn above on the basis of analysis of excitation spectra. At the illumination stage related to the plateau in  $E_{x440}/E_{x490}$ , the observed decrease of fluorescence seems not to be exclusively dependent on a particular pool of pigments. The period of illumination of photosynthetic apparatus in which the here discussed molecular mechanisms undergo (approx. 4–5 min) may be correlated with light-in-

duced conformational changes, reported of taking place in LHCII [24]. Those changes affecting potentially mutual orientation and intermolecular distance between antenna pigments, might be in a high probability considered as inducing the energetic uncoupling between xanthophyll pigments and chlorophylls, reported above. According to this kind of explanation the xanthophyll-related fluorescence decrease should be a result of a decreased rate of excitation flow to chlorophylls, related to the affected distance-dependent and orientation-dependent [18] process of intermolecular energy transfer. Another kind of explanation of an energetic uncoupling of a certain pool of xanthophyll pigments from photosynthetic processes may be based on a simple detaching of these antenna pigments from pigment-proteins. This mechanistic model has a support from the studies on the xanthophyll cycle [25–27]. There exists the light-dependent limitation in violaxanthin de-epoxidation in the xanthophyll cycle termed as violaxanthin availability [25–27]. The process of making violaxanthin available to de-epoxidation may be understood as providing the protein-bound *in situ* pigment molecules with a more diffusional freedom in order to facilitate direct contact with a membrane-anchored de-epoxidase enzyme [28–30]. The molecular process reported in this paper, and referred to above as an energetic uncoupling should be the natural consequence and a spectroscopic manifestation of a reduced direct contact between the antenna system and this particular pool of xanthophylls. The present study does not classify this pool of pigments as violaxanthin but such a hypothesis seems to be reasonable, taking into account exceptionally weak bounding of violaxanthin to the thylakoid pigment-proteins, manifested by a relatively high presence of this pigment, in particular, in a free-pigment fraction in every kind of preparations of pigment-protein complexes [20]. On the other hand, the energetic coupling of exogenous violaxanthin to isolated antenna complex can be easily demonstrated (see Fig. 3).

In conclusion, we report here the physiological mechanism demonstrated by an energetic uncoupling of a certain xanthophyll pool from antenna chlorophylls. This mechanism was found to be particularly active between 100 s and 300 s of illumination of photosynthetic apparatus *in vivo*. We

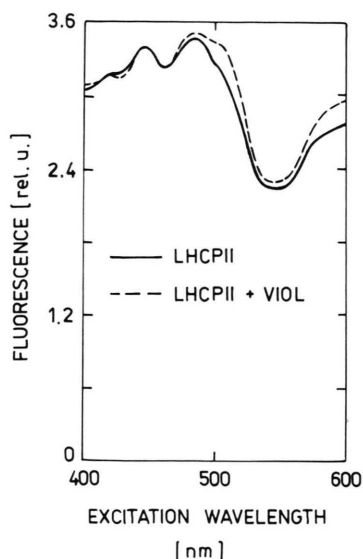


Fig. 3. Excitation spectra (emission at 680 nm) of LHCII preparation (—) and of the same sample after modification with an exogenous violaxanthin (---). Spectra were normalized at 440 nm. For more experimental details see text.

hypothesize that this molecular mechanism is related to the process of making violaxanthin available to de-epoxidation in the xanthophyll cycle, which precedes zeaxanthin formation. During the experiments presented in Fig. 2 the absorption

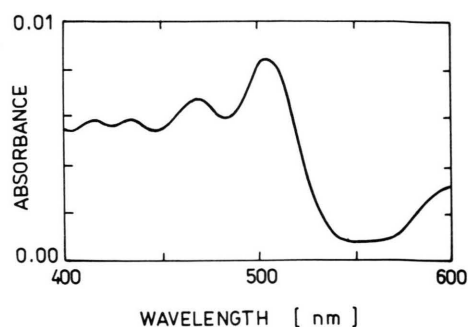


Fig. 4. Difference absorption spectrum of the intact rye leaf: illuminated for 10 min (20 W/m<sup>2</sup>) minus the same leaf before the light treatment.

spectrum of the examined leaf changed as demonstrated in Fig. 4. The prominent increase in the absorbance in the region of 505 nm is a manifestation of activity of violaxanthin de-epoxidation in the leaf-sample during illumination [25–27]. In fact, violaxanthin to zeaxanthin ratio evaluated in such a leaf is 4.8, before the illumination decreased to a value of 1.3, as determined after 10 min of light treatment in conditions described in Figs. 1, 2 and 4.

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