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Energy transfer process during senescence: fluorescence and photoacoustic studies of intact pea leaves

Wiesław I. Gruszecki *, Konka Veeranjaneyulu **, Bogumil Zelent and Roger M. Leblanc

Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, Trois-Rivières (Canada)

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In the present study, the time-course of changes in fluorescence and in in vivo photochemical activities, from full expansion through senescence, have been studied in third-pair leaflets of pea (*Pisum sativum* cv. Early Dwarf). The ratio between the fluorescence intensities observed at 680 nm for excitation wavelengths of 475 nm and 600 nm respectively, decreased around the 20th day, indicating a decreased energy transfer from carotenoids to chlorophylls in Photosystem II. Photoacoustically monitored photosynthetic oxygen evolution and photochemical energy storage decreased in parallel with the decrease in the fluorescence intensity ratio. In contrast, no decline was seen until the 25th day in the ratio between the intensity of fluorescence emission at 730 nm for this same excitation wavelength pair. This suggests that the energy transfer mechanism is affected first in Photosystem II and only later in Photosystem I. The decrease in the photothermal component preceded the massive destruction of photosynthetic pigments. The ratio of fluorescence emission at 685 nm to 730 nm with excitation at 450 nm increased after a massive decrease in pigment content of leaves. Furthermore, fluorescence emission at 600 nm, possibly due to carotenoids, and the ratio of fluorescence intensity at 600 nm to 685 nm, increased during the course of leaf senescence.

Introduction

Leaf senescence is characterized by a decline in photosynthesis [1,2]. It has been reported that senescence induces alterations in chloroplast structure and function. These alterations include a decline in photosynthetic pigment content, thylakoid membrane disorganization and a decrease in photochemical activity [1,3–7]. A few in vitro studies on partial reactions of photosynthesis in the presence of electron acceptors and donors indicated a deterioration in electron transport [8–11], possibly due to changes in the levels of electron transport carriers [1,12]. Recently, it has been demonstrated that senescence alters the accessibility of the electron transport chain to exogenous electron acceptors and donors, and causes a shift in the pH optima of photochemical activity. This acidic shift in the pH optima of photochemical activity during senescence [13–15], further supports the changes in the structural and functional integrity of membrane system [16]. However, the sequential changes leading to loss of photochemical activity are not clearly understood. Any change in membrane organization can also affect the primary events of photosynthesis. Limited information is available on changes in the energy transfer process during senescence, hence we attempted to understand the changes in the energy transfer process and the in vivo photochemical activities of an intact pea leaf using fluorescence and photoacoustic methodologies.

Photoacoustic spectroscopy (PAS), a relatively recent technique, permits the study of in vivo photochemical energy storage and photosynthetic O_2 evolution of an intact green leaf. It involves the detection of modulated heat and O_2 pulses from leaves irradiated with intensity modulated light. Both heat and oxygen periodically diffuse from the chloroplast to the cell surface, where their modulated pulses are transduced into acoustic waves. These waves are transmitted through the epidermal layer and detected by a microphone in the sealed

* Present address: Institute of Physics, Maria Curie-Skłodowska University, Lublin, Poland.

** Permanent address: Department of Botany, Sri Krishnadevaraya University, Anantapur, India.

Abbreviations: PAS, photoacoustic spectroscopy; PES photochemical energy storage.

Correspondence: R.M. Leblanc, Centre de recherche en photobiophysique, Université du Québec à Trois-Rivières, 3351, boul. des Forges, C.P. 500, Trois-Rivières (Québec), G9A 5H7, Canada.

photoacoustic cell [17]. Because of its unique features, such as sample non-destructivity and insensitivity of the O_2 signal to stomatal closure and respiration, we employed PAS to follow the changes in the in vivo photochemical activities, from full expansion through senescence of an intact pea leaf.

Materials and Methods

Pea (*Pisum sativum* L. cv. Early Dwarf) plants were raised from seeds in pots containing organic earth, vermiculite and sand (3 : 1 : 1). Seedlings from uniformly germinated seeds were maintained in each pot in a growth room with a photoperiod of 14 h, light intensity 90 $W \cdot m^{-2}$, temperature $23 \pm 2°C$ and relative humidity 55–60%. Plants were watered with NPK fertilizer (Plant-Prod 20-20-20, Plant Products, Bramalea, Ontario), to a point slightly in excess of field capacity. Third-pair leaflets appeared between the 9th and 10th day, and were fully expanded between the 13th and 14th day from the date of sowing. Henceforth, as used in the text, the number of days mentioned corresponds to the day after sowing. Measurements were always made with an 18 mm leaf disk punched towards the apical region in the third-pair leaflets and data are the average of measurements on three leaves. From the average value, the data dispersed not more than 10%.

Fluorescence excitation and emission spectra were recorded by means of a Spex Fluorolog 2 (Metuchen, NJ) spectrofluorometer equipped with Datamate DM1 data acquisition system and a water cooled Hamamatsu Model R928 photomultiplier tube. Fluorescence was detected in frontal geometry. Excitation spectra were corrected for the lamp intensity. Fluorescence lifetime measurements were performed using a Photochemical Research Associates, Model 3 000 (PRA, London, Ontario) fluorescence lifetime single photon counting instrument. The sample was illuminated in the spectral region of 440 ± 50 nm, and fluorescence emission was detected at 680 ± 5 nm. Data were analyzed by a PRA statistical deconvolution program.

Photoacoustic measurements were made with a home-built photoacoustic spectrometer, as described earlier [18]. A light beam supplied by a 1000 W xenon lamp was passed through a monochromator (Schoeffel Instrument Corporation, Westwood, NJ). The monochromatic beam (680 nm, 12 $W \cdot m^{-2}$) was modulated by a mechanical chopper and directed onto the sample placed in a closed photoacoustic cell. The background non-modulated saturating light (250 $W \cdot m^{-2}$) was directed onto the sample by using a fiber-optic light guide. The acoustic signal was detected by a microphone, preamplified and then analyzed by a lock-in amplifier (Ithaco Dynatrac, Model 393, Ithaca, NY). The amplifier was operated in the two phase mode, in order to record simultaneously the (vectorial) in-phase and quadrature components. The amplitudes of the photothermal (A_{PT}) and oxygen (A_{OX}) signals were evaluated according to Ref. 17. Photochemical energy storage was measured at a high frequency of 405 Hz, where, only the photothermal signal exists. Photoacoustic spectra were recorded in the presence and in the absence, of non-modulated background light between 400 and 750 nm.

Chlorophylls and carotenoids were extracted using 80% acetone and the levels were estimated according to Ref. 19.

Results and Discussion

The simple and direct proof of excitation energy transfer from carotenoids to chlorophylls[10] * is the contribution of the light absorbed by carotenoids[9] in chlorophyll fluorescence[2]. It is possible to follow any changes in this process by analyzing the chlorophyll fluorescence excitation spectra of intact pea leaves. The typical fluorescence excitation spectra are presented in Fig. 2a. The carotenoid band presence in the chlorophyll fluorescence excitation spectrum can be quantitatively characterized by the ratio of the intensity in the excitation spectrum at 475 nm to the intensity at 600 nm. At shorter wavelengths (475 nm) both chlorophylls and carotenoids, and at longer wavelengths (600 nm) only chlorophylls are responsible for the changes in absorption, hence reflecting the changes in the excitation spectrum.

The intensity ratio, as a parameter linked to the carotenoid-chlorophyll singlet-singlet excitation energy transfer, is plotted against the age of the plant in Fig. 3. In both panels of Fig. 3, the same dependence is plotted at conditions for excitation spectra registered at different wavelengths of fluorescence emission: 730 nm (Fig. 3a) and 680 nm (Fig. 3b). Since the former position corresponds to the fluorescence of Photosystem I (PS I) and the latter to Photosystem II (PS II), it is possible to monitor differences in energy transfer during aging of the two photosystems. As we can see, at around the 20th day, energy transfer[10] decreases in the pigments connected to the PS II light-harvesting complex. The partial disappearance of the carotenoid band in the chlorophyll excitation spectrum can be attributed to the 'disconnection' of carotenoids from chlorophylls. This mechanism referred to here as 'disconnection' is presumably a process affecting the chlorophyll-carotenoid spatial location, and can be related to certain conformational changes in the pigment-protein complexes. Fig. 3a shows that a comparable change is not observed in

* The numbers refer to the photophysical processes schematically depicted in Fig. 1.

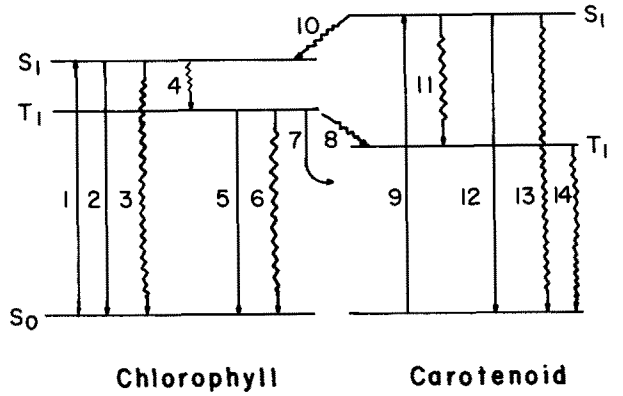

Fig. 1. Schematic representation of the photophysical processes which occur in the light-harvesting pigment molecule system (first excited singlet and triplet states are considered only): (1) light absorption by chlorophyll; (2) chlorophyll fluorescence; (3) chlorophyll singlet non-radiative deactivation; (4) intersystem-crossing from the excited singlet to the triplet state of the chlorophyll; (5) chlorophyll phosphorescence; (6) chlorophyll triplet non-radiative deactivation; (7) chlorophyll triplet quenching by other processes, for example photosensitization-singlet oxygen formation; (8) chlorophyll-carotenoid triplet-triplet energy transfer; (9) light absorption by carotenoid; (10) carotenoid-chlorophyll singlet-singlet energy transfer; (11) intersystem crossing from the excited singlet to the triplet state of the carotenoid; (12) carotenoid fluorescence; (13) and (14): deactivation of the excited states of a carotenoid in non-radiative processes. S_0, T_1 and S_1 denote the ground state, first excited triplet state and first excited singlet state, respectively. The energy levels are an arbitrary scale.

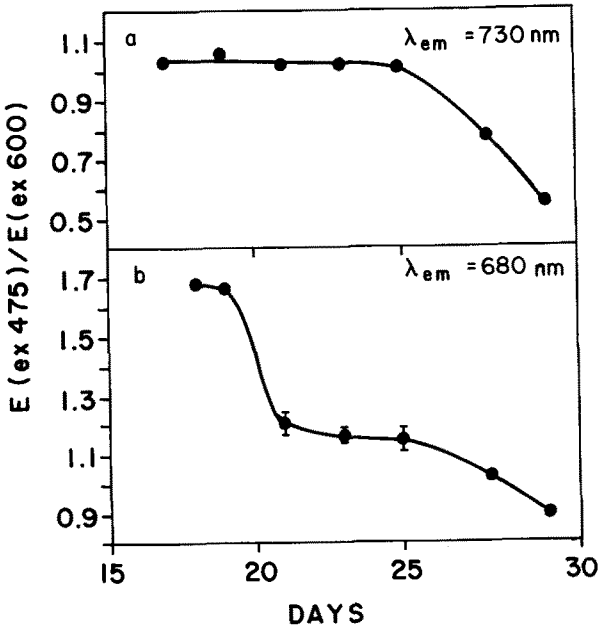

Fig. 3. The ratio of intensity at 475 nm to 600 nm in the fluorescence excitation spectra of intact pea leaves over time, where $\lambda_{em} = 730$ (a) and $\lambda_{em} = 680$ (b).

PS I around the 20th day, since the intensity ratio measured at 730 nm remains unchanged.

The partial loss of carotenoid-chlorophyll energy transfer can be correlated to similar changes in photoacoustically monitored oxygen evolution (A_{OX}), and photochemical energy storage (PES) of the intact leaf (Fig. 4b and c). Our finding that the energy transfer mechanism related to PS II is affected first during senescence does not agree with some earlier reports [10,20] where the photosynthetic activity of PS I was affected before that of PS II. Such a sequence of events is probably the result of a lower stability, in course of aging, of the stroma thylakoids [5,6] which are known to be enriched with PS I particles [21]. A comparison of these reports with our findings suggests that the observed carotenoid-chlorophyll 'disconnection' in the light-harvesting pigments, associated with PS II at about the 20th day, is not related to massive pigment-protein breakdown. It can more accurately be interpreted as a result of some very subtle mechanisms changing the protein surface, and affecting the pigment molecular organization. The proposed explanation is supported by the fact that this change is not accompanied by significant destruction of photosynthetic pigments (Fig. 5), particularly the chlorophyll *b* known to be present

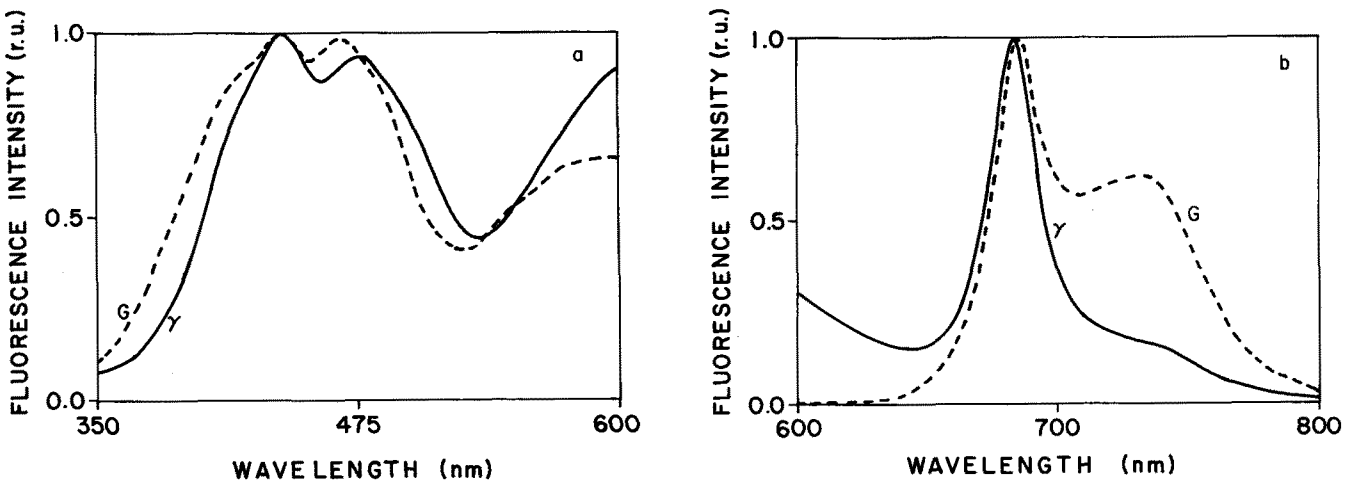

Fig. 2. Fluorescence excitation (a) and emission (b) spectra of intact pea leaves. ------ green (G) 17th day; ——— yellow (Y) 28th day. The excitation and the emission spectra were normalized at 438 nm ($\lambda_{em} = 680$) and 684 nm ($\lambda_{ex} = 450$), respectively.

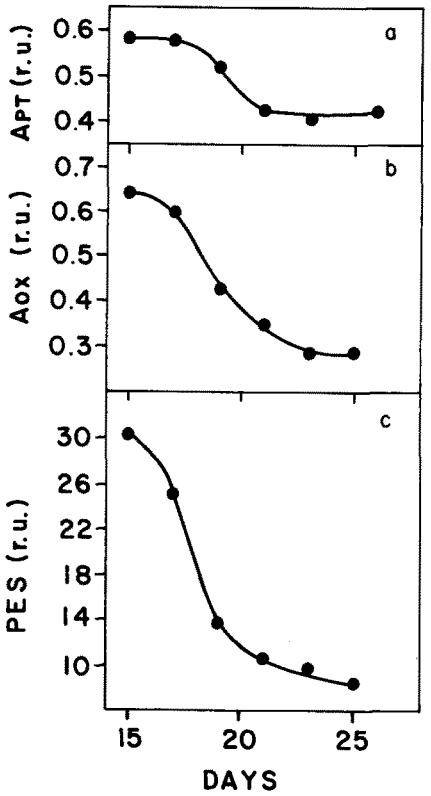

Fig. 4. Amplitude of photothermal signal (a), oxygen signal (b), and photochemical energy storage (c) of intact pea leaves over time. Analytical modulated light: λ, 680 nm; intensity, 12 $W \cdot m^{-2}$; frequency, 25 Hz.

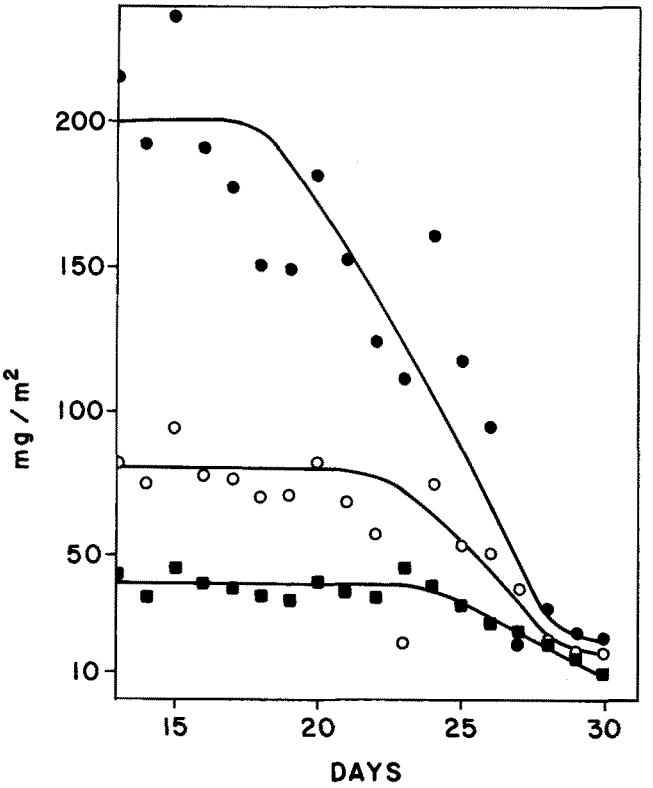

Fig. 5. Levels of chloroplast pigments in course of time in pea leaves. ●, chlorophyll *a*; ○, chlorophyll *b*; ■, carotenoids.

mainly in PS II-associated pigment-proteins [22]. Additional support for the above proposed mechanism comes from our findings on high-light treated intact pea leaves, where the carotenoid-chlorophyll 'disconnection' phenomenon can be reversibly observed (unpublished results). A further decrease in the above parameters seen after the 25th day, combined with the extensive photosynthetic pigment breakdown, is most likely related to irreversible membrane degradation.

The above mechanism of carotenoid disconnection from photosynthetic activity was also clearly demonstrated by the photoacoustic spectra of the green and

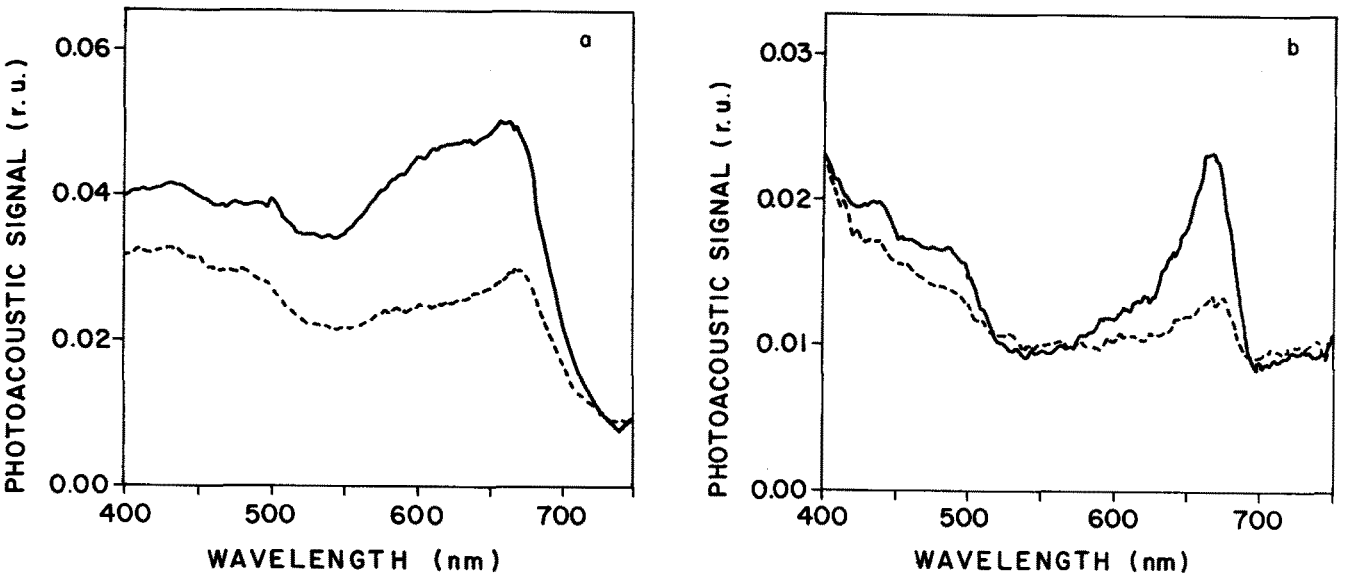

Fig. 6. Photoacoustic spectra of intact pea leaves registered in the presence (- - - - -), and the absence (———) of non-modulated background light (250 $W \cdot m^{-2}$). Modulated light: frequency, 80 Hz; intensity at 680 nm, 12 $W \cdot m^{-2}$. (a) green leaf; (b) senescent leaf.

senescent leaves (Fig. 6). These spectra were registered, in the presence (dotted line) and in the absence (solid line) of strong, non-modulated background light that saturates photosynthesis. The difference in the signal amplitudes between the two spectra indicates the relative levels of photosynthetic O_2 evolution. As can be seen, both in green as well as in senescent leaf, chlorophylls are active in photosynthetic O_2 evolution. This is demonstrated by the large decrease of the photoacoustic signal in the presence of non-modulated background light in the 680 nm region, where the chlorophylls are active as light-absorbing pigments. The decrease in acoustic signal in the presence of background light is, however, not so intense in the short-wavelength region of the photoacoustic spectra of senescent leaf, when compared to green leaf. In this region, both chlorophylls and carotenoids are active, and so the lack of an effect must be attributed to the absence of carotenoid pigments in the photosynthetic action spectrum. Since the light absorbed by carotenoids cannot be transferred to the chlorophyll component of the energy transfer chain, these pigments cannot participate in oxygen evolution. This is clearly evident from the small decrease of photoacoustic signal in the presence of non-modulated background light, in the short wavelength region. This effect is more clearly pronounced in the region between 520 and 570 nm, where chlorophylls do not absorb light, but carotenoids are active especially in the aggregated form [23,24]. Although there is no direct evidence to state that native photosynthetic pigments are in the aggregate form, the very strict limitations imposed on the distance criteria in energy transfer processes [25,26], requires a close packing of pigments, which can be interpreted as aggregation.

As Fig. 4 shows, all the reported photoacoustic parameters (A_{PT}, A_{OX}, PES) evaluated with 680 nm modulated light decrease in the course of aging. A few mechanisms can be considered as responsible for such an effect. One possible explanation is the decrease in the concentration of the light-absorbing pigments. However, the different time-dependences of the changes in pigment levels and photoacoustic parameters strongly discount this as the main mechanism. Another explanation can be considered, according to which a decrease in thermal energy dissipation[3] is connected to a parallel increase of fluorescence[2]. The fluorescence quantum yield is known to be strongly dependent on the pigment concentration. The fluorescence, which is quenched in highly organized molecular assemblies can increase during the course of the partial pigment destruction. In fact, we have noticed an increase in chlorophyll fluorescence at 680 nm, between the 18th and 20th day (Fig. 7a). Our observation that the decrease of the photothermal component (Fig. 4a) is followed by massive photosynthetic pigment bleaching, but not vice versa, may suggest another possible explanation. The loss of

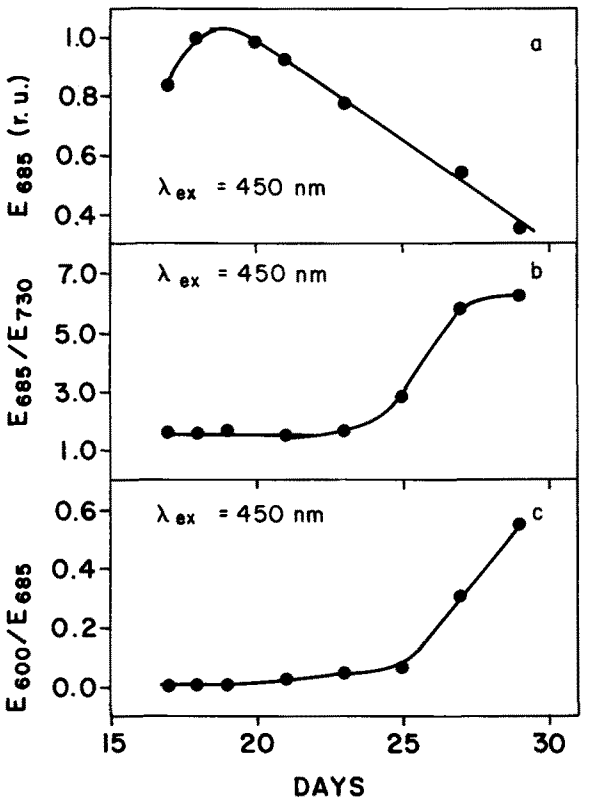

Fig. 7. Parameters extracted from fluorescence emission spectra of intact pea leaves over time. Excitation wavelength is 450 nm; (a) emission intensity at 685 nm; (b) the ratio of emission intensity at 685 nm to 730 nm; (c) the ratio of emission intensity at 600 nm to 685 nm.

the singlet-singlet carotenoid-chlorophyll energy transfer[10] is most probably achieved by structural changes in the functional protein-pigment complexes. These changes can affect the intermolecular distance, which in turn affects the excitation energy transfer [25–28]. The triplet-triplet energy transfer can also be affected by the same mechanism as discussed for singlet-singlet energy transfer. The singlet-singlet energy transfer from carotenoids to chlorophylls[10] is responsible for the antennae function of the yellow pigments in vivo, and the triplet-triplet energy transfer from chlorophylls to carotenoids[8] is considered in plant cells to be the photoprotective mechanism [29,30]. The loss of this process results in chlorophyll photobleaching, as observed in the present study (Fig. 5). The main mechanism of pigment destruction is known to be the oxidation of chlorophyll by photo-sensitized singlet oxygen and free radicals [29,30]. Photosensitization is a process, which competes with the other channels of chlorophyll deactivation, and should decrease the stream of energy emitted as heat. This is another very probable mechanism causing the observed decrease of the photothermal component of the photoacoustic signal during senescence.

Another phenomenon connected with the qualitative changes in the in vivo chlorophyll fluorescence is usually reported in plants under stress conditions [31–34].

This change involves the preferential disappearance of the fluorescence emission at 730 nm. This phenomenon can also be observed in the present study by comparing the fluorescence emission spectra of green and senescent leaves (Fig. 2b); it can be quantitatively analyzed using the fluorescence emission intensity ratio of 685 nm to 730 nm (Fig. 7b). The increase in the ratio has generally been attributed to the reabsorption of the fluorescence and its decrease after partial chlorophyll destruction [33,34]. The fact that the fluorescence ratio does not change in parallel with the pigment breakdown process, and in fact starts in the last part of its phase [31–33] (compare also Fig. 5 and Fig. 7b), suggests that there are other responsible mechanisms. The 730 nm fluorescence is mainly attributed to the PS-I-associated chlorophyll; therefore its variation can be related to changes in the molecular organization of the PS I pigment-protein complex. The ratio of fluorescence intensity at 685 nm to 730 nm can be correlated to changes in the energy transfer processes linked to PS I (compare Fig. 7b and Fig. 3a).

Another interpretation of the qualitative changes in the fluorescence emission spectra can be proposed based on the work with chlorophyll in different model systems. It is well known that dissolved chlorophyll has its main fluorescence maximum at around 680 nm. This can be observed when the pigment is dissolved in organic solvents, surrounded by the lipid molecules in liposomes, or in monomolecular or multimolecular layers [35]. The fluorescence spectra are changed drastically when the ethanolic chlorophyll solution is hydrated. The pigments form the aggregates, with a broad fluorescence band, with a maximum at approximately 730 nm (unpublished). The increase of fluorescence intensity in the spectral region above 700 nm can also be observed by examining chlorophyll in molecular assemblies in Langmuir-Blodgett films [36]. Assuming that the decrease in chlorophyll fluorescence intensity at 730 nm is an expression of the chlorophyll disaggregation, one can interpret the senescence dependent change in the ratio of intensity at 685 nm to 730 nm as a projection of the molecular assemblies' disintegration. Such an interpretation obtains strong support from the comparative fluorescence kinetic study of intact green and senescent leaves (Fig. 8). The chlorophyll fluorescence decay (Fig. 8a) is described using the monoexponential kinetic, with the characteristic time constant $\tau_0 = 0.28 \pm 0.02$ ns. Similar results have been reported previously [37]. How-

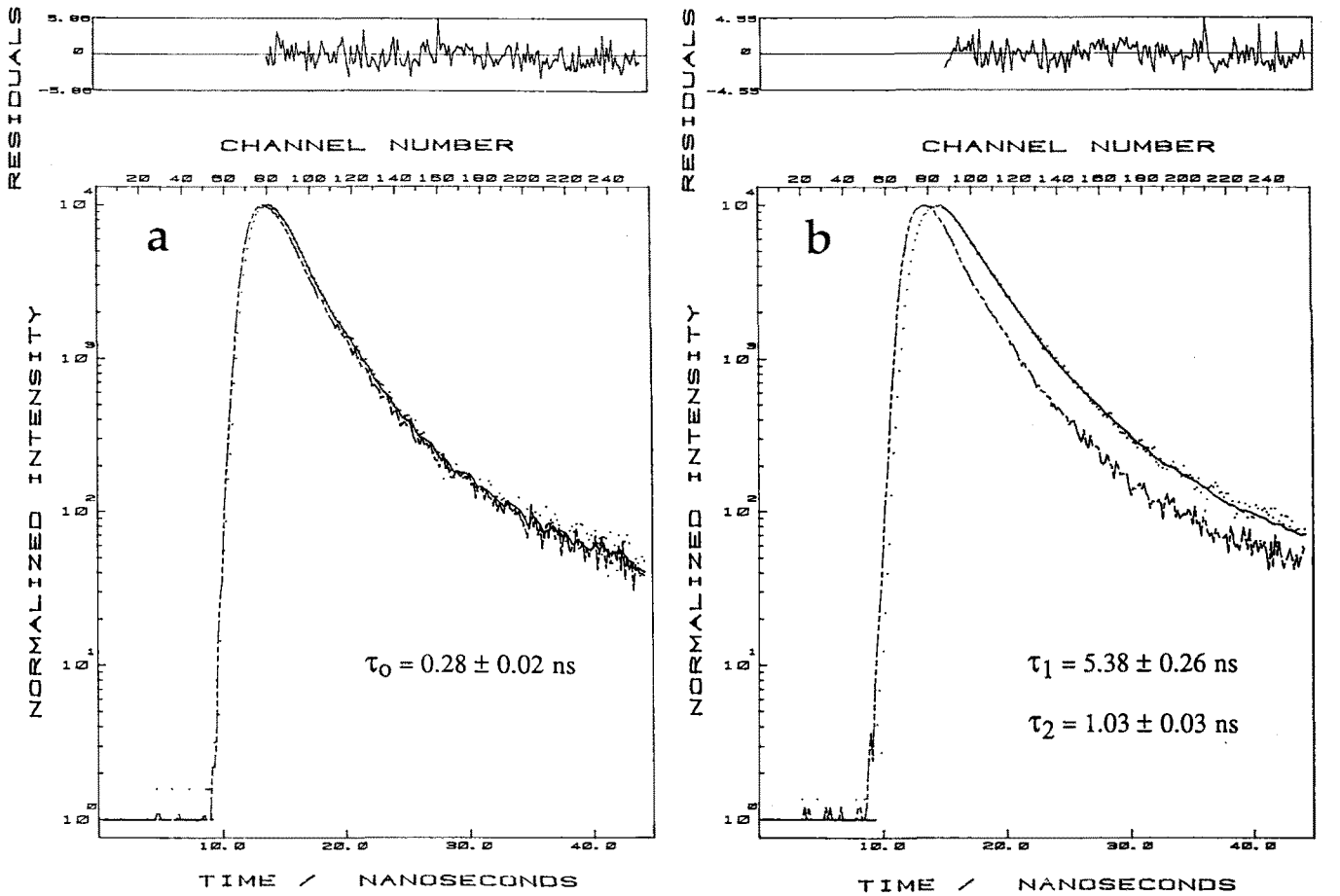

Fig. 8. Kinetics of the decay of chlorophyll fluorescence in intact pea leaves. (a) 17th day green leaf; (b) 28th day senescent leaf. Flash (- - - - - -) and fluorescence (· · · · · ·) profiles.

ever, in some reports, the in vivo chlorophyll fluorescence kinetics was described using two- and three-exponential approaches [34,38]. The characteristic time of chlorophyll fluorescence decay in the senescent leaf is evidently longer, as seen in Fig. 8b, and fits a two exponential approach; $\tau_1 = 5.38 \pm 0.26$ (10%), $\tau_2 = 1.03 \pm 0.03$ (90%). Knowing that the characteristic time constant of the fluorescence of the molecules is directly linked [35,39] to the aggregation state, we can interpret the observed change as a proof of the disintegration of chlorophyll assemblies.

A more detailed analysis of the intact leaf fluorescence spectra (Fig. 2a) points out changes in the spectral region below 650 nm. Such changes were reported earlier in [34], and were called as the fluorescence of 'some other pigments'. The fact that this band appears only in the region where the carotenoid fluorescence has recently been reported [40–43], suggests that the radiative deactivation of the yellow pigments is responsible for the enhancement in fluorescence of senescent leaf. The increase in the fluorescence intensity in the carotenoid region can be expressed by the intensity ratio at 600 nm to 685 nm. The change in this parameter in the course of aging is depicted in Fig. 7c, with an increase in the ratio generally observed after the 19th day. One can correlate this fact with the above discussed loss of singlet-singlet energy transfer from the carotenoids to chlorophylls[10]. This phenomenon of the loss of carotenoid singlet state quenching by the neighboring chlorophylls, potentially increases the probability of carotenoid singlet deactivation by other processes including fluorescence[12]. The proposed mechanism is much more pronounced after massive green pigment bleaching after 25th day, as seen by comparing Figs. 5 and 7c.

Considering the above discussed mechanisms, the following conclusions can be drawn regarding the molecular organization and energy transfer processes during senescence. Molecular organization of the light-harvesting complex associated to PS II is affected first. This alters the energy transfer efficiency from carotenoids to chlorophyll pigments resulting a decrease in photochemical activity. The carotenoid-chlorophyll 'disconnection' is also related to the loss of triplet-triplet energy transfer. Since this mechanism is important in photoprotection, its loss is accompanied by the photobleaching of the photosynthetic pigments.

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