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A new type of thermoluminometer: A highly sensitive tool in applied photosynthesis research and plant stress physiology[a]

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Summary

Here we describe a newly developed thermoluminescence measuring device that employs flash excitation, peltier heating, and light detection by channel photomultipliers (CPM). The new thermoluminometer is equipped with four sample holders for simultaneous measurements of thermoinduced light emission in the temperature range from −20 °C to +180 °C. It allows one to measure leaf samples, chloroplasts, thylakoids, algae, or even bioorganic material lacking chlorophyll by means of naturally induced or artificially applied chemilumigenic probes. The temperature range of the thermoluminometer allows one to analyse the thermoinduced radical pair recombination of photosystem II in the lower temperature region as well as chemiluminescence from lipid peroxidation in the higher temperature region. Hence, plant material can be assessed concerning both its photosynthetic and its oxidative stress status. Since the device is equipped with four sample holders and four CPM channels for simultaneous detection of thermoinduced light emission, it facilitates a high throughput. Therefore, the new device is interesting, not only in ecophysiology, but also in the field of plant breeding, as it can be used to study the stress tolerance of various cultivars of cultural crop plants.

Key words: algae – chemiluminescence – heavy metals – herbicides – hydrobiology – lipid peroxidation – ozone – plant breeding – plant stress physiology – PS II – thermoluminescence – UV – water-splitting apparatus

Abbreviations: AG band = Afterglow-band. – B_1 band = TL band induced by the recombination of the radical pair $S_3Q_B^-$. – B_2 band = TL-band induced by the recombination of the radical pair $S_2Q_B^-$. – C-band = TL-band induced by the recombination of the radical pair $Y_D^+Q_A^-$. – CL-band = chemiluminescence band. – DCMU = 3(3,4-dichloro-phenyl)–1,1-dimethylurea. – DMSO = dimethyl-sulfoxide. – HTL = High temperature TL band. – Q-band = TL-band induced by the recombination of the radical pair $S_2Q_A^-$. – TL = thermoluminescence. – T_{peak} = peak temperature of a respective TL band

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Introduction

The photosynthetic apparatus as target for environmental stress

Oxygenic photosynthesis plays an important role for primary production in aquatic as well as terrestrial ecosystems. The estimation of primary production from phytoplankton and higher plants is a crucial factor in the study of ecosystems and in the field of agriculture. Photosynthesis, as the leading physiological process that determines primary production, is significantly influenced by environmental stress factors. Several routine methods, such as gas-exchange and different chlorophyll fluorescence techniques, are applied to measure the efficiency of the photosynthetic apparatus under environmental stress conditions (Schreiber and Bilger 1987). Thermoluminescence, another method in photosynthesis research, has recently been used for studying the influence of stress factors on the photosynthetic apparatus in phytoplankton or higher plants (Ducruet and Vavilin 1998, Gilbert et al. 1998, Misra et al. 2001, Ducruet 2003, Skotnica et al. 2003, Gilbert et al. 2004). However, thermoluminescence measurements are not routinely applied in this field although they show remarkable advantages compared to conventional gas-exchange and chlorophyll-fluorescence techniques. Thermoluminescence glow curves can monitor different physiological states of photosystem II, as well as the oxidative stress level, that is, the accumulation of lipid peroxidation products.

Like fluorescence, TL in the temperature range from 0–60 °C originates specifically from Photosystem II. However, while chlorophyll fluorescence monitors the quenching of excitation energy in the antenna system prior to charge separation, TL provides a differential insight into charge separation and stabilization of PS II, thereby providing detailed information about the donor as well as the acceptor side (Inoue 1996). This complexity of information can be used for stress-selective monitoring.

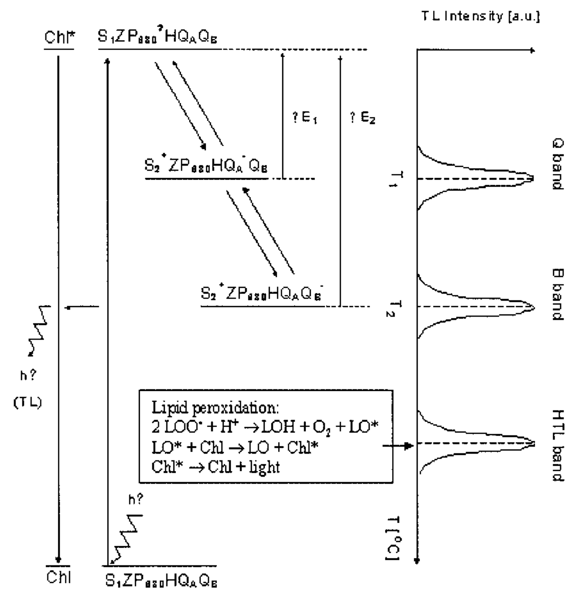

Figure 1. Energetic scheme of TL emission generated by charge recombination in PS II (detailed description in text): S_2 = positively charged S state of the water splitting apparatus formed after flash excitation from the S states S_0 and S_1, Z = redox active tyrosine Y_Z, P680 = Primary donor of PS II reaction center, H = primary electron acceptor pheophytin, Q_A = primary quinone acceptor, Q_B = secondary quinone acceptor, Chl = Chlorophyll, $h\nu$ = light excitation, ΔE_1 = activation energy barrier between the radical pair state $S_2Q_A^-$ and the excited state P_{680}^*, ΔE_2 = activation energy barrier between the radical pair state $S_2Q_B^-$ and the excited state P_{680}^*, Lipid peroxidation (exemplarily): $LOO^{\cdot}$ = lipid peroxyl radical, LO^* = excited lipid carbonyl triplet, LOH = lipid alkoxyl.

Characteristics and origin of different TL bands

Light absorption in Photosytem II reaction centers results in the excitation of the primary donor, $P_{680} \rightarrow P_{680}^*$. Subsequently, primary charge separation and electron transfer processes lead to abstraction of electrons from water within the water splitting apparatus (S states) and transfer of electrons to the secondary quinone acceptor Q_B (Fig. 1). Concerning the S states, the description will be restricted to the S_2 and S_3 state, because these are the only charged S states that can recombine with electrons on the primary or secondary quinones. In the presence of PS II herbicides, electron transfer from Q_A to Q_B is inhibited and hence electrons can be stabilized on the primary quinone acceptor Q_A. The resulting radical pairs $S_2Q_A^-$ (Rubin and Venediktov 1969, Rutherford et al. 1982) and $S_{2/3}Q_B^-$ (Rutherford et al. 1982) are energetically stabilized and have a barrier of activation energy against recombination [E_A ($S_{2/3}Q_B^-$) > E_A ($S_2Q_A^-$)]. At room temperature the thermal activation is high enough to supplement this free energy loss and spontaneous charge recombination of these radical pairs takes place leading to re-excitation of P_{680}. The re-excited primary donor in turn can decay to the ground state by emitting luminescence, the so-called delayed fluorescence (Strehler and Arnold 1951). However, at lower temperatures the light-induced radical pairs are metastable and charge recombination must be thermally stimulated by heat transfer. In this case the induced luminescence is called thermoluminescence resulting in characteristic glow curves with typical peak maxima at different temperatures for the $S_2Q_A^-$ (Q-band) and $S_{2/3}Q_B^-$ (B-band) radical pairs [T_{peak} ($S_{2/3}Q_B^-$) > T_{peak} ($S_2Q_A^-$)]. Beside the $S_2Q_A^-$ and $S_{2/3}Q_B^-$ radical pair state, further radical pair states of the PS II reaction center can contribute to TL glow curves (Table 1) (for reviews, see Sane and Rutherford 1986, Demeter and Govindjee 1989, Vass and Inoue 1992, Inoue 1996, Vass and Govindjee 1996, Ducruet 2003, Tyystjärvi and Vass 2003).

The shape and the peak position of the different TL bands provide valuable information about the energetic stability of the respective radical pair and about the functioning of the PS II reaction center. Different environmental stress factors or

Table 1. Thermoluminescence bands in photosynthetic samples. AG = Afterglow; CL = Chemiluminescence; HTL = High temperature TL band; T_{max} = peak temperature.

Name	Origin	T_{max}	Reference
Q band	$S_2Q_A^-$ recombination	0 °C – 10 °C	(1)
B band	$S_{2/3}Q_B^-$ recombination	20 °C – 30 °C	(1)
C band	$Y_D^+Q_A^-$ recombination Typically stimulated when Q_B is inhibited by herbicides or PSII is damaged by stress. Y_D is the non-functional donor to $P680^+$, Y_Z is the functional donor.	50 °C – 55 °C	(1)
AG band	Afterglow (AG): induced by reverse electron flow to TL-inactive S_2Q_B states generating TL-active $S_2Q_B^-$ radical pair states.	40 °C – 45 °C	(2 – 3)
HTL1 (CL)	Aldehydes + H_2O_2?, particularly stimulated in wet samples	60 °C – 90 °C	(4) – (7)
HTL2 (CL)	Lipid peroxides	120 °C – 140 °C	(4 – 5), (8 – 9)
HTL3 (CL)	Heat induced autooxidation of membranes	> 160 °C	(4 – 5)

References: (1) Vass und Inoue 1992; (2) Miranda und Ducruet 1995; (3) Roman and Ducruet 2000; (4) Ducruet 2003; (5) Vavilin und Ducruet 1998; (6) Hideg und Vass 1993; (7) Stallaert et al. 1995; (8) Vavilin et al. 1998; (9) Merzlyak et al. 1992.

chemical compounds can interact specifically with certain sites of the PS II reaction center or the photosynthetic electron transport chain (Vass and Inoue 1992). Even small changes in the redox properties of different radical pairs concerning either their donor or acceptor side species or both will be reflected in the pattern of TL glow curves (Horvath 1986, Demeter and Govindjee 1989, Vass and Govindjee 1996). In consequence, the changed pattern of the TL glow curves can deliver a fingerprint concerning the effects of abiotic and biotic stress factors on plants.

In the high temperature range above 60 °C the thermoinduced light emission is no longer induced by radical pair recombination processes in PS II, but is instead induced by chemiluminescence (CL) due to radiative thermolysis of lipid peroxidation products (Vavilin and Ducruet 1998). Several high temperature TL bands, HTL1, 2, and 3, have been described and attributed to triplet carbonyls and singlet oxygen induced during lipid peroxidation and lipid hydroperoxide or lipid cycloperoxide thermolysis (Vavilin and Ducruet 1998, Ducruet 2003, Havaux 2003). These excited species transfer their excitation energy to chlorophylls, which emit chemiluminescence. In particular, the amplitude of the HTL2 band correlates well with the concentration of lipid hydroperoxides or thiobarbituric acid reactive substances estimated by biochemical methods (Vavilin and Ducruet 1998, Havaux and Niyogi 1999, Havaux et al. 2003). The extent of lipid peroxidative damage (i. e. the amount of chemiluminescence emitting molecules) depends on the equilibrium among lipid peroxide formation, the state of the antioxidative system, the quenching of excess excitation energy, and the repair of damaged lipids. The characteristic of this equilibrium is indicative of the stress severity and the plant potential to resist stress. In the field of oxidative stress commercially available chemiluminometers are often used to measure the ultraweak chemiluminescence from organic tissues at constant room temperature (Stanley and Kricka 1991). Since chemiluminescence is strongly stimulated by increasing the temperature, thermoluminometry can provide a much higher sensitivity in this special field of research.

Materials and Methods

Description of the new thermoluminometer system

The measuring device is composed of the following components as can be seen in Figure 2:

1. Sample unit,
2. Sample holder, consisting of a copper plate with 4 sample places (Ø = 0.8 cm),
3. Temperature sensor integrated in the copper plate (S201PD, Telemeter Electronic),
4. Peltier plates in direct contact to the sample holder for heating and cooling (HT 6-12-40 and HT 8-12-40, Melcor), temperature ranging from –20 °C to +180 °C,
5. Flash unit including two photo single-turn-over flash lamps (25 µs),
6. Channel photomultiplier unit with 4 integrated CPMs (C 973, Perkin-Elmer),
7. Filter holder to select the emission waveband by different cut off filters. The standard filter is a RG 650 (Schott) to select specifically the red chlorophyll luminescence,
8. Motor driven thread rod for positioning of the flash- and CPM unit,
9. Tubing connectors for flushing the sample compartment with nitrogen or other artificial gas compositions,
10. Main control unit including, for example, peltier driver, flash driver, and analog digital converter,
11. PC including a MS-Windows control and analysis software (FAN TL 1.0) based on Labview hard- and software components (NI 6023, differential inputs with 12 bit, National Instruments Corporation). The user-friendly software allows one to change a variety of parameters, such as the time of dark adaptation, the temperature at different program steps, the flash number, the delay before the temperature gradient is started, and the temperature gradient, including the starting/ending temperature and the heating rate. Hence, users can easily develop their own excitation and temperature programs for specialized applications.

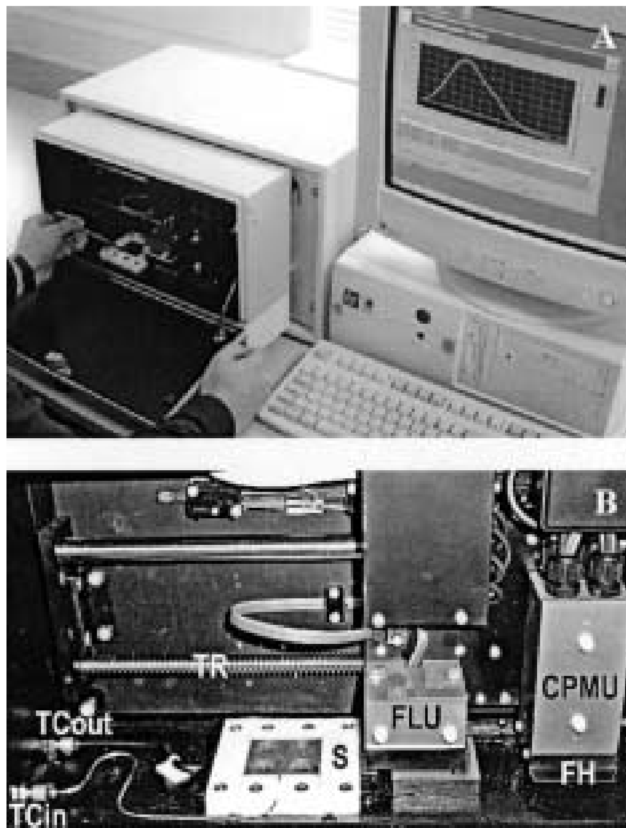

Figure 2. The newly developed thermoluminometer: A, view on the set-up with opened sample unit together with PC displaying a set of TL glow curves; B, view into the sample unit, CPMU = Channel photomultiplier unit with 4 separate CPMS, FH = Filter holder with a RG 650 filter (Schott, Germany) in place, FLU = Flash unit with single turn over flash lamps, S = Sample holder with 4 sample places ∅ = 0.8 cm, TCin = Tubing connector inlet for application with artificial gas compositions, TCout = Tubing connector outlet, TR = Thread rod for moterdriven positioning of the flash and CPM unit.

The samples holders can be filled with suspensions of chloroplasts, thylakoids, or microalgae. Solid samples like leaf pieces or phytoplankton samples on glass fibre filters are pressed with steel or teflon rings to the copper surface. When temperatures above 80 °C are applied, samples should be transferred to filters to avoid boiling of large liquid volumes. In the case of plant material, the standard procedure for TL measurements is programmed and automatically executed in the following steps: First a dark adaptation for 5 min at room temperature is performed to establish a defined ratio of the S states (S_0/S_1 = 25 : 75) and oxidized to reduced Q_B ($Q_B : Q_B^-$ = 50 : 50; Inoue 1996). Then the sample is cooled to +2 °C or –15 °C. Simultaneously, the flash unit (FLU, Fig. 2 B) is positioned automatically by the motor driven thread rod above the sample holder and an excitation by one or more single-turn-over flashes is triggered. Next the CPM unit (CPMU, Fig. 2 B) is moved above the sample holder. The heating gradient (20 °C min^{-1}) is started immediately and the thermoinduced light emission from the samples is recorded independently by 4 CPMs (Fig. 3 A).

In the case of Q band measurements ($S_2Q_A^-$), the samples are infiltrated with PS II herbicides, such as DCMU, and cooled to –15 °C before flash excitation (Fig. 3 B). A rather low temperature is needed to avoid spontaneous recombination of the $S_2Q_A^-$ radical pair, which is characterized by a lower activation energy barrier than the energetically more strongly stabilized $S_{2/3}Q_B^-$ radical pair (Fig. 1).

Measurements of the B band ($S_{2/3}Q_B^-$) can be performed by flash excitation under non-freezing conditions at 2 °C providing sufficient stability of this radical pair against spontaneous recombination (Fig. 3 A). The sample compartment is equipped with tubing connectors to flush the sample compartment with different gas compositions. For measurements in the high temperature region, flushing with N_2 will avoid chemiluminescence induced by autooxidation of the sample (HTL3, Vavilin and Ducruet 1998). Hence, only the accumulation of lipid peroxidation products induced by oxidative stress under physiological temperatures will be detected. In the low temperature region different concentrations of oxygen and CO_2 can be applied to study their effect on the radical pair dynamics in PS II.

Plant material

Barley [*Hordeum vulgare* L. convar. Distichon (L.) Alef. s. L. var. nutans (Rode) Alef, «Abed Bomi», A-HOR4512, Denmark] and tomato plants [*Lycopersicon esculentum* Mill. covar. parvibaccatum Lehm. var. cerasiforme (Dun.) Alef. s. L., «Benarys Gartenfreude», LYC 17/81] were grown in a plant culture room under a light dark cycle of 16 h light and 8 h dark at an intensity of 200 µmol m^{-2} s^{-1} (PAR) and a temperature of 24 °C ± 1 °C. Relative humidity was kept at 60 % ± 5 %. Barley was used after a growing period of 10 days and tomatoes after 4 weeks. In barley the primary leaves were used and samples of 0.8 cm length were cut 2 cm underneath the leaf tip for TL measurements. Leaf samples of tomatoes have been punched with a cork drill (diameter 0.8 cm) from the 4th leaf storey counted from the cotyledons. *Chlorella vulgaris* [strain 211–11 b, SAG, Göttingen, Bolds Basal Medium (Schlösser, 1982)] was grown in aerated batch cultures in a light-dark rhythm of 16/8 hours at 60 µE m^{-2} s^{-1} and 20 °C. The algal cultures were grown for 5–6 days up to a chlorophyll concentration of 5–6 µg ml^{-1}.

Chlorophyll determination

Chlorophyll concentration was determined by spectrophotometry after pigment extraction in 80 % acetone according to Jeffrey and Humphrey (1975).

Thylakoid preparation

Thylakoids from dark adapted pea plants were prepared as described elsewhere (Chow and Anderson 1987) with minor modifications. Thylakoids (60 µg Chl/mL) were measured in a reaction medium (330 mmol/L Sorbitol, 10 mmol/L NaCl, 5 mmol/L $MgCl_2$ and 40 mmol/L HEPES pH = 7.6) containing 500 units catalase, to convert H_2O_2 into H_2O, and 100 µmol/L methylviologen as a terminal electron acceptor. A stable artificial ΔpH in the dark was established in the thylakoid suspension by ATP hydrolysis with 0.3 mmol/L ATP (Gilmore and Yamamoto 1992). The ATPase was activated by a short actinic light period of 3 min (500 µmol photons/m^{-2} s^{-1}).

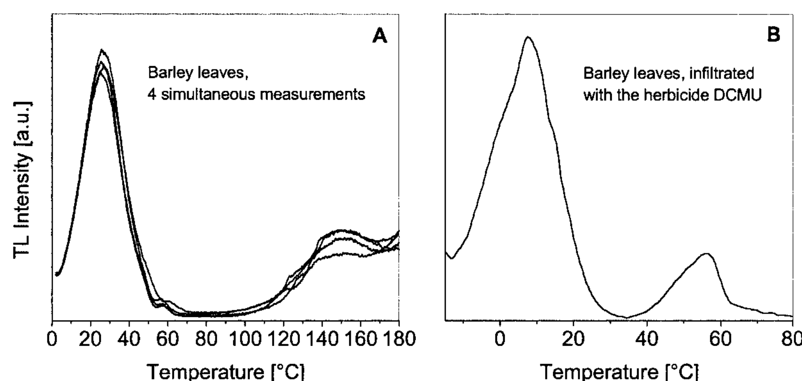

Figure 3. A: 4 simultaneously recorded TL curves of 4 barley leaf samples measured independently with the 4 channels of the CPM unit. Leaf samples were taken from the light and dark adapted for 5 min at 20 °C, cooled to 2 °C and excited with one single turn over flash. Immediately after flash excitation the temperature gradient (20 °C min^{-1}) was started and the thermoinduced light emission was recorded by the CPM units. Bands: 24 °C, B band ($S_2Q_B^-$); 55 °C, C band ($Y_D^+Q_A^-$); 150 °C, HTL2 band (lipid peroxides). **B:** barley leaf samples vacuum infiltrated with 50 µmol/L DCMU (averaged curves, n = 4). The pretreatments were the same as in Fig. 3 A, except that the samples were cooled to –15 °C before flash excitation. Bands: 7 °C, Q band ($S_2Q_A^-$); 55 °C, C band ($Y_D^+Q_A$).

UV irradiation

UV radiation was provided by 3 fluorescent tubes (TL 40 W/12, Philips), which were operated for at least 100 h prior to use. The UV radiation was filtered by 2 layers of cellulose diacetate films (N125, Pütz-Folien, Taunusstein Wehen, Germany) to remove any UV-C radiation (< 280 nm). The cellulose acetate films were replaced all 4 h because of ageing. The intensity of UV-B (280–315 nm) was measured with an integrating UV radiometer (Krochmann, Berlin, Germany) and adjusted to 1.8 W m^{-2}.

Ozone fumigation

Tomato and barley plants were incubated in an ozone chamber (rectangular plexiglass box) at 250 ppb of ozone for 6 h at a temperature of 22 ± 1 °C and a relative humidity of 80 ± 5 %. The photosynthetic photon flux density on top of the plants was adjusted to 200 µmol m^{-2} s^{-1} PAR. The ozone chamber was continuously ventilated with a regulated flow of ozone-fumigated air that was charcoal-filtered before. Ozone was produced by a mercury vapour lamp. The concentration of ozone in the chamber was analysed by an ozone detector ML 9810 (Monitor-Labs, USA).

Herbicide treatment

Atrazine (2-ethylamino-4-chloro-6-isopropylamino-1,3,5-triazine) and diuron [3(3,4-dichloro-phenyl)-1,1-dimethylurea] were purchased by Aldrich-Sigma. Stock solutions of herbicides were prepared in DMSO. The final solvent concentration was 1 % (v/v). Barley leaves were vacuum infiltrated with 100 µmol/L DCMU for 3 min. *Chlorella* cultures were dark-adapted for 12 h and diluted to 1 µg mL^{-1} in culture medium before herbicide treatment. 40 mL of the diluted culture, including the final atrazine concentration, were incubated for 20 min in the dark and afterwards homogenously filtered on glass-fibre filters (GF6, Schleicher & Schüll, Dassel, Germany). Controls were treated in the same way with 1 % DMSO containing no herbicides. The prepared filters, containing 40 µg chlorophyll a, were directly used for TL measurements.

Brain slices from rats

Slices of the prefrontal cortex were obtained from male Wistar rats. Slices were prepared and maintained as previously described (Brand et al. 1999, Eschke et al. 2001). A block of the rat brain including the pyramidal cells of the prefrontal cortex was rapidly removed after decapitation. Five coronal slices with a thickness of 200 µm were cut with a vibratome and transferred to an interface holding chamber containing cold artificial cerebrospinal fluid. Individual slices were transferred to the sample holders. 10 µl of a chlorophyll pigment extract (2 mg Chl *a* mL^{-1}) were added on top of the brain slices. Oxidative stress was induced by illumination of the chlorophyll infiltrated brain slices with white light (3000 µE m^{-2}, 1 min).

Application in photosynthesis research

While variable chlorophyll fluorescence monitors primarily the acceptor side related reduction of Q_A (Schreiber et al. 1998), TL is an excellent technique to study donor as well as acceptor side related changes of PS II in detail (Vass and Inoue 1992). For acceptor side related aspects, see the chapter «Application in hydrobiology».

Especially complex is the operation of the donor side where 4 electrons have to be extracted in sequence from the manganese cluster of the water oxidizing complex for water splitting and evolution of oxygen. Five different S states, S_1 to S_4, of the water splitting apparatus have been described (Haumann and Junge 1994, Inoue 1996). The differential detection of the S_2 and S_3 states by means of TL measurements allows one to study S state cycling when a sequence of single turn over flashes is applied. Hence, TL can help to localize changes in the functioning of the water splitting apparatus at the $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$, and $S_3 \rightarrow S_4(S_0)$ transitions and can provide important information about the role of different inorganic cofactors or extrinsic proteins, such as Mn, Cl^- and Ca^{2+} or

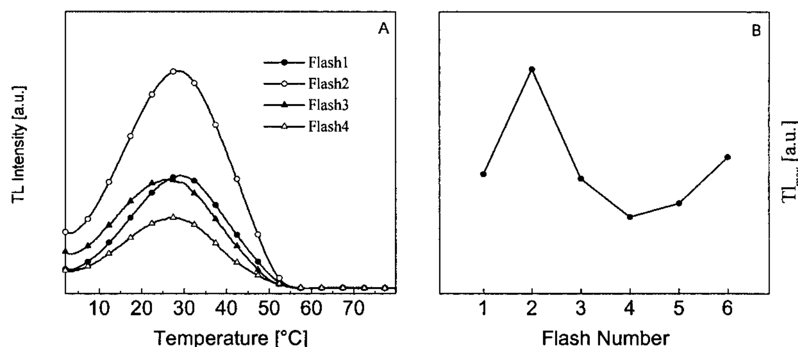

Figure 4. A: TL curves from suspensions of pea thylakoids (60 µg Chl a) with an artificially induced proton gradient (300 µmol/L ATP, 100 µmol/L methylviologen). Thylakoids were excited at 2 °C with 1, 2, 3 and 4 single turn over flashes. The changes in the amplitude and the peak position of the B band show the S state cycling. After 3 flashes the B band is down shifted to a peak position of 26 °C compared to 29 °C at one flash ($p < 0.001$, $n = 8$). **B:** Period-four oscillation of TL B band amplitude in thylakoids (as described in Fig. 4 A) under illumination with a series of single turn over flashes (averaged values, $n = 8$).

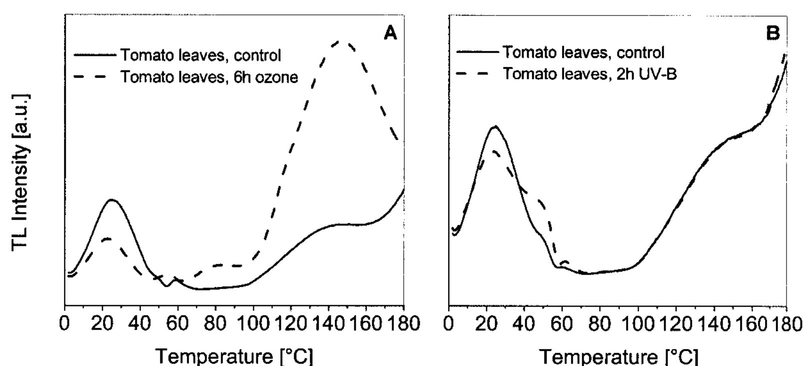

Figure 5. A: TL curves of tomato leaf samples with and without ozone fumigation (250 ppb, 6 h). Pretreatments as in Figure 3 A (averaged curves, $n = 6$). Bands: 23 °C, B band ($S_2Q_B^-$); 55 °C, C band ($Y_D^+Q_A^-$); 60 °C, ?; 80 °C, HTL1 band (aldehydes + H_2O_2), 150 °C, HTL2 band (lipid peroxides). **B:** tomato leaf samples before and after UV-B irradiation (1.8 W m^{-2}, 2 h) (averaged curves $n = 6$). Bands: 23 °C, B band ($S_2Q_B^-$); 40–55 °C, AG band and/or C band ($Y_D^+Q_A^-$); 60 °C, ?; 80 °C, HTL1 band (aldehydes + H_2O_2), 150 °C, HTL2 band (lipid peroxides).

the 16–33 kDa proteins (for review see Vass and Inoue 1992). Figure 4 shows the S state cycling reflected in the change of the B band depending on the flash number for thylakoids with an artificially induced pH gradient. In briefly dark adapted thylakoids, the B band shows a period four oscillation in intensity with maxima after the second and the sixth single turn over flash (Fig. 4 B). This S state pattern is determined by the ratios of S_0/S_1 and Q_B/Q_B^- at the beginning of the flash sequence as well as by the different TL quantum yield of the $S_2Q_B^-$ and $S_3Q_B^-$ radical pair state ($S_3Q_B^-$: $S_2Q_B^-$ = 1.7–2.0; Rutherford et al. 1984, Demeter et al. 1985; for review see Inoue 1996). During the flash sequence, not only the TL intensity is changing but also the peak temperature of the B band is varying (Fig. 4 A). Below a lumenal pH of 7.0, the B band exhibits two distinguishable components with a few degrees difference (Inoue 1981). The B_1 band is generated by recombination of the radical pair state $S_3Q_B^-$ while the B_2 band represents the recombination of $S_2Q_B^-$. Since the S_3 state is destabilized more strongly at high proton concentrations than is the S_2 state, the B_1 band peaks at lower temperatures than does the B_2 band (Rutherford et al. 1984). The peak shifting can be well observed by comparison of the TL glow curves in briefly dark adapted thylakoids after one and three single turn over flashes (Fig. 4 A). After one single turn over flash, pure $S_2Q_B^-$ radical pairs are generating a B band with higher peak temperature. In the case of three flashes, the $S_3Q_B^-$ radical pairs dominate, yielding a B band with a lower peak temperature (c. f. Inoue 1996).

Application in plant stress physiology

Various stress factors can damage the photosynthetic apparatus or can induce oxidative stress leading to lipid peroxidation. Hence, TL measurements can provide a highly informative matrix for monitoring the functional state of PS II together with the membrane integrity.

Figure 5 shows the effect of ozone fumigation (Fig. 5 A) and UV-B irradiation (Fig. 5 B) on tomato leaves. The amplitude of the B-band ($S_2Q_B^-$) at 23 °C decreases by almost 50 % after

ozone fumigation and the peak temperature shifts a few degrees to lower values. At 55 °C a TL band is enhanced that most likely represents a C band generated by recombination of the radical pair $Y_D^+Q_A^-$ (Demeter et al. 1984, Sane and Rutherford 1986, Vass and Inoue 1992). The low temperature region clearly displays that the stress factor ozone strongly affects charge separation and radical pair dynamics in PS II. In the high temperature region, ozone treatment intensifies the TL emission between 60–180 °C by 200 %. Most tremendous is the increase between 100 °C–160 °C, the temperature range of the HTL2 band representing lipid peroxidation (Vavilin and Ducruet 1998, 2003). The consequence of this oxidative stress is the induction of visible damage in the form of leaf necrosis 3 days later (data not shown). The effect of ozone is not a direct reaction of this gas with cell compounds, but rather ozone triggers a so-called oxidative burst concomitant with the enzymatic generation of reactive oxygen species (ROS) that can lead to the so-called hypersensitive response (HR) (Wohlgemuth et al. 2002). TL glow curves of ozone treated leaves provide evidence that TL is an excellent method to monitor simultaneously PSII integrity as well as the oxidative stress level of leaves. Furthermore, TL measurements can predict strong damage to plants several days before visible damage occurs. A more detailed description of ozone effects on TL glow curves in comparison to chlorophyll fluorescence measurements is provided in Skotnica et al. (2003).

The effect of UV-B irradiation on the B band in tomato leaves lead to a loss of only 15 % concerning its amplitude. No significant changes are observed between 60–180 °C. However, a significant shoulder at 50 °C is observed that might represent again a C-band ($Y_D^+Q_A^-$) enhanced by UV-B stress. A contribution of an after glow band generated by reverse electron flow is also possible (Table 1, Miranda and Ducruet 1995). Despite the fact that UV-B irradiation can cause oxidative stress (Strid et al. 1994), the tested tomato variety seems to be stress tolerant in this respect. The comparison of both TL glow curves (Fig. 5 A, B) shows that tomato leaves are much more sensitive to the stress factor ozone than to the stress factor UV-B.

Another interesting field of plant stress physiology includes biotic stress factors. Rahoutei et al. (1999) could demonstrate that tabac-mosaic virus infection of plants changes the characteristics of TL glow curves.

Application in hydrobiology

The potential of TL to analyse the integrity of the photosynthetic apparatus and the oxidative stress status can also be applied to phytoplankton algae. Beside natural environmental factors, like light, UV-B, and temperature, the influence of different water pollutants, such as herbicides or heavy metals are of special interest for TL measurements in the field of hydrobiology. These compounds interact highly specifically with the donor and/or the acceptor side of PS II. Small shifts in the redox energy of different radical pairs concerning either their donor or acceptor side species or both can classify chemical compounds according to their donor or acceptor side action in PS II.

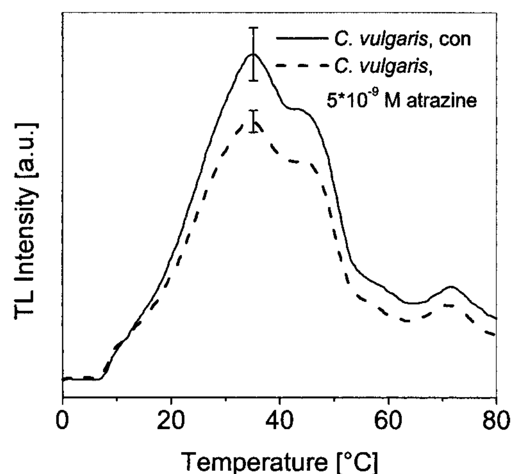

Figure 6. TL curves from *Chlorella vulgaris* (40 µg Chl a on glass fibre filters) with and without the PS II herbicide atrazine (averaged curves, n = 8, error bars represent standard deviation). Pretreatment as described in Fig. 3A. Bands: 30 °C, B band ($S_2Q_B^-$); 45 °C, AG band?; 70 °C, HTL1 band.

Different chemical classes of PS II herbicides, such as triazines (atrazine), phenylureas (diuron, isoproturon), phenolics, triazinones and uraciles, are applied in weed control and contaminate lakes and water reservoirs. Since PS II herbicides act as competitive inhibitors at the Q_B binding site, their action results in a decrease of the B band amplitude (Fig. 6) and in an increase of the Q band. Due to different interaction of certain herbicides from various chemical classes with the Q_B binding site in the PS II reaction center, not only the microenvironment of the Q_B-binding site but also that of the Q_A-binding site is changed, thereby modulating its redox properties (Koike et al. 1989). In consequence, the temperature peak of the Q-band is down-shifted about 12 °C–15 °C in the case of phenolic herbicides, like ioxynil or bromoxynil, compared to phenylurea-type herbicides or triazines (Vass and Demeter 1982, Horvath 1986, Koike et al. 1989). Hence, TL measurements of the Q-band can identify the dominating class of PS II herbicides in respective water samples.

Heavy metals are another class of chemical compounds that interact specifically with the PS II reaction center and influence the character of TL glow curves. Some agricultural herbicides or algicides represent, for example, copper salts. The effect of heavy metals at lower concentrations on the B-band of TL glow curves has been described by several authors (Mohanty et al. 1989, Vass and Inoue 1992, Horvath et al. 1998) and is primarily restricted to the B_2-band ($S_2Q_B^-$). The B_2-band is largely inhibited at lower concentrations of heavy metals like Cu^{2+} (Horvath et al. 1998) resulting in a pro-

Table 2. Influence of different stress factors on TL bands. A = Amplitude; P = change of the peak position of the respective TL band; upright arrow = increase in the amplitude or up-shift in the peak position to higher temperatures; down-right arrow = decrease in the amplitude or down-shift in the peak position to lower temperatures; – = no changes.

	Photo-inhibition	UV	Ozone	PSII-Herbicides	Heavy metals	Salt stress	Drought stress	Heat stress
Q band	A↓	A↓	?	A↑, P↓↑[1]	A-	A↓	?	A↓
B band	A↓, P↓	A↓	A↓,P↓	A↓	A↓, P↓	A↓	A↓	A↓
C band	A-	A↑	A↑	A↑	A-	?	?	A-
AG band	?	?	?	?	?	A↑	A↓	A↓
HTL bands	A↑	A(↑)	A↑	?	A↑	?	?	A-[2]
Reference	1–5	6–8	9	10–11	5, 12–13	14–16	17	4–5, 17–18

[1] The peak position of the Q band strongly depends on the type of PS II herbicide that is applied [c. f. (11)].

[2] No changes in HTL bands are observed up to 50 °C, above 50 °C an increase in HTL2 occurs [c. f. (5)]. References: (1) Ohad et al. 1988; (2) Vass et al. 1988; (3–4) Misra et al. 1997, 1998; (5) Vavilin et al. 1998; (6) Hideg et al. 1993; (7) Gilbert et al. 1998; (8) Turcsanyi 2000; (9) Skotnica et al. 2003; (10) Horvath 1986; (11) Vass and Demeter 1982; (12) Mohanty et al. 1989; (13) Horvath et al 1998; (14) Sahu et al. 1998; (15) Misra et al. 1999; (16) Krieger et al. 1998; (17) Janda et al. 1999; (18) Joshi et al. 1995.

nounced decrease at the right wing of the total B-band and hence in an apparent downshift of the whole B-band ($B_1 + B_2$) concerning its peak temperature. Since PS II herbicides will decrease the B_1- and B_2-band in parallel, the effect of those compounds could be clearly distinguished from heavy metal effects when several single turn-over flashes are applied to yield a mixture of $S_2Q_B^-$ and $S_3Q_B^-$. Therefore, TL measurements provide the chance to detect beside PSII herbicides also heavy metal pollution.

An overview of the influence of different stress factors on TL signatures in various plant material is given in Table 2.

The changes of TL curves due to different stress factors show that the pattern of TL bands between –15 °C to +180 °C might serve as a stress specific fingerprint in the analysis of plants. However, as various question marks indicate, further work is needed to develop TL measurements into a reliable tool in plant stress research and ecophysiology.

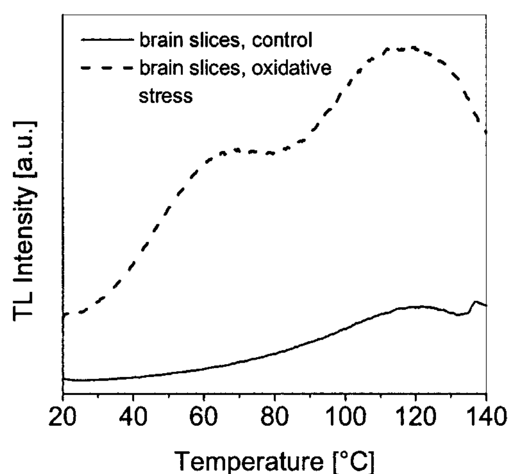

Figure 7. TL curves from cortical brain slices from rats infiltrated with an acetonic chlorophyll solution with and without white light from a Schott lamp (Schott, Germany) at 3000 µE m^{-2} for 1 min. The strong illumination triggered oxidative stress by singlet oxygen formation via light induced triplet chlorophyll states. Samples were dark adapted for 5 min at 20 °C and heated with 20 °C min^{-1} from 20 °C to 140 °C without flash excitation (chemiluminescence bands). Bands: 70 °C, HTL1 (aldehydes + H_2O_2); 115 °C, HTL2 band (lipid peroxides). Representative tracings of brain slices taken from the same rat.

Other potential applications

Lipid peroxidation is also the dominating process triggered by oxidative stress in non-photosynthetic tissue of plants (Mathew and Roy 1992) and animals (Kobayashi et al. 1999). The membrane peroxidative event proceeds via a free-radical chain reaction in the course of which singlet oxygen ($^1O_2$) and excited triplet carbonyls are generated as chemiluminescent species. $^1O_2$ shows emission maxima in the red at 634 and 703 nm, while triplet carbonyls emit in the blue-green wavelength range between 450–550 nm (Sies 1987). The amplitude of this particular spectral emission is well correlated with peroxide concentration (Miyazawa et al. 1994) or that of malondialdehyde (Doi et al. 2002), emphasizing that chemiluminescence can be used to monitor lipid peroxidative damage. Miyazawa et al. (1994) have applied thermoluminescence successfully in the field of food chemistry for the rapid estimation of peroxide content in soybean oil. Since the thermolytic breakdown of certain lipidperoxides stimulates the generation of chemiluminescent triplet carbonyls and $^1O_2$, thermoluminometry can strongly enhance the sensitivity of chemiluminescence measurements.

There is increasing evidence that reactive oxygen species (ROS) and lipid peroxidation are involved in the damage of cells in different organs, including the brain, such as cerebrovascular diseases dealing with hypoxic/ischemic tissue damage (Dirnagl et al. 1995). In particular, the temporal pattern

and the cellular sources of ROS production in ischemia/reperfusion of the brain are unknown (Schreiber et al. 1995). Chemilumigenic probes, like luminol or lucigenin, react specifically with H_2O_2 and O_2^- in conjunction with light emission (Li et al. 1995). Hence, TL might provide both a highly sensitive technique to detect lipid peroxidative damage and the generation of short-lived ROS.

Figure 7 shows a first attempt to study the effect of oxidative stress in cortical brain slices of rats that have been infiltrated with a chlorophyll pigment extract as a sensitizer. The illumination with strong light induces the formation of triplet chlorophyll states that are deactivated via the generation of highly oxidative singlet oxygen (Asada and Takahashi 1987). The light induced oxidative stress causes an eight-fold increase between 60–90 °C (HTL1) and a five-fold increase between 90–140 °C (HTL2). The latter represent, as discussed earlier, an excellent indicator of oxidative stress.

Conclusions

The newly developed thermoluminometer is equipped with four separate sample holders for simultaneous measurements of thermoinduced light emission from different bioorganic materials. On the basis of this high sample throughput, the new measuring device is particularly interesting for applied fields of photosynthesis research, like plant stress physiology or hydrobiology. The large temperature range from –20 °C to 180 °C of the thermoluminometer allows one to monitor the integrity of the photosynthetic apparatus in the low temperature region (–20 °C to +60 °C) as well as the oxidative stress level in the high temperature region (60 °C to 180 °C). Both parameters are important indicators for the stress tolerance of plants. While the photosynthetic capacity determines the primary productivity, the level of oxidative stress allows one to predict if chronic damage will occur. Therefore, the newly developed TL device is also interesting for the field of plant breeding. Since a large variety of new cultivars can be produced by modern molecular techniques, efficient and fast methods for the estimation of stress resistance are needed to reduce the amount of less resistant and less promising cultivars in time consuming but still necessary field studies. TL measurements can provide this fast estimate of stress tolerance in new cultural crop varieties. Beyond the field of plant research, the new TL device can be used to measure chemiluminescence. In particular, lipid peroxidative damage and the generation of ROS by means of chemilumigenic probes can also be monitored in non-photosynthetic tissue.

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