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REVIEW ARTICLE: FIELD TECHNIQUES

Chlorophyll thermoluminescence of leaf discs: simple instruments and progress in signal interpretation open the way to new ecophysiological indicators

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Abstract

Luminescence from photosynthetic material observed in darkness following illumination is a delayed fluorescence produced by a recombination of charge pairs stored in photosystem II, i.e. the back-reaction of photosynthetic charge separation. Thermoluminescence (TL) is a technique consisting of a rapid cooling followed by the progressive warming of a preilluminated sample to reveal the different types of charge pairs as successive emission bands, which are resolved better than the corresponding decay phases recorded at constant temperatures. Progress in thermoelectric Peltier elements and in compact light detectors made the development of simple, affordable and transportable instruments possible. These instruments take advantage of multifurcated light guides for combined TL, fluorescence and absorbance/reflectance measurements. Meanwhile, experiments on unfrozen leaf discs, with excitation by single turn-over flashes or far red light and infiltration by specific inhibitors/uncouplers, have led to a better understanding of *in vivo* TL signals. Much like chlorophyll fluorescence and in a complementary way, TL in the 0–60 °C temperature range not only informs on the state of photosystem II in leaf tissues and its possible alterations, but also gives a broader insight into the energetic state inside the chloroplast by probing (1) the light-induced or dark-stable thylakoid proton gradient through the protonation of the Mn oxygen-evolving complex, (2) the induction of cyclic/chlororespiratory electron flow towards the plastoquinone pool, (3) the [NADPH+ATP] assimilatory potential. By a different mechanism, warming above 60 °C without preillumination reveals chemiluminescence high temperature thermoluminescence (HTL) bands due to the radiative thermolysis of peroxides, which are indicators of oxidative stress in leaves.

Key words: Delayed fluorescence, instrument, luminescence, photosynthesis, photosystem II.

Introduction

Oxygenic photosynthesis is endowed with the unique property of a variable fluorescence emission, F_v spanning from a minimum level F_0 to an approximately 5-fold higher maximum level F_m. It originates from chlorophylls *a* in the photosystem II antenna. In the mid 1980s, the introduction of light-doubling experiments, then of chlorophyll fluorimeters using a modulated excitation and saturating light pulses, allowed the separation of the two mechanisms of F_m quenching that contribute to this variable emission by (i) a photochemical quenching, due to the ability of 'open' PSII centres to trap the energy of an absorbed photon conveyed to them through the antenna to create a +/– charge pair: these centres become temporarily 'closed', so they cannot trap the incoming energy which has to be dissipated in the pigment antenna partly as fluorescence emission, and (ii) a non-photochemical quenching, which reduces the excess excitation energy in

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Abbreviations: A band, Z^+Qa^-; AG, afterglow (luminescence bounce); B band, S_2Qb^- (B2) and S_3Qb^- (B1); C band, D^+Qa^-; DLE, delayed light emission; HTL, high temperature thermoluminescence (> 60 °C); LED, light emitting diodes; PSII, photosystem II; PSI, photosystem I; PMT, photomultiplier tube; TL, thermoluminescence; T_m, temperature of the maximum of a TL band (here corresponding to a 0.5 °C s^{-1} heating rate).

the antenna in situations where absorbed light overcomes the photosynthetic capacity (e.g. on a cold sunny day).

At the onset of a dark period following a strong illumination, a weak 'luminescence' emission, approximately 1% of the fluorescence intensity, can be detected with an emission spectrum similar to that of chlorophyll fluorescence (for reviews, see Lavorel, 1975; Jursinic, 1986; Tyysjärvi and Vass, 2003). At a constant temperature, this emission follows (in theory) a multiexponential decay kinetics. It results, with a low yield, from the recombination of charge pairs generated by prior illumination. This recombination recreates a singlet excited state in the chlorophyll antenna, which is partly dissipated as luminescence, also called 'delayed' fluorescence by reference to 'prompt' fluorescence emission observed during an illumination (the term 'phosphorescence' used for triplet state emissions is not relevant here). Photosynthesis is possible because light-separated charge pairs are stabilized on electron carriers by activation energy barriers, which limit the back-reaction. However, the sum (stabilized energy+activation energy) corresponds to the quantum energy of red photons, so the height of the activation barrier is necessarily limited and can be considered optimized for the physiological temperature domains of various photosynthetic organisms. Thus, charge recombination, reflected by luminescence emission, is highly temperature-dependent and grows exponentially according to an Arrhenius law up to 50–60 °C, a temperature range where the heat-induced breakdown of PSII occurs. As a consequence, thermoluminescence is a technique particularly well-adapted to study luminescence: it consists of cooling the sample before or immediately after an actinic illumination to make the recombination rate negligibly slow, then in warming it progressively in order to reveal the various types of luminescence-emitting charge pairs as TL elementary bands. This technique has a better resolving capacity than luminescence decays (LD) recorded at constant temperature, being composed of several exponential and non-exponential phases. Each elementary TL band corresponds to an exponential LD phase.

Compared with chlorophyll fluorescence which reflects the quenching of photonic energy in the antenna prior to charge separation, luminescence provides original information on the energetics of the charge stabilization system, beyond the photochemical trap. Since the 1960s, these two techniques have been used successfully to build a model of PSII, later refined by biochemical, genetic and spectroscopic methods and ultimately by crystallography. There was global confirmation of the PSII model by TL in the early 1980s (Rutherford *et al*., 1982, 1984*a*, *b*; Demeter and Vass, 1984; Rutherford and Inoue, 1984; Hideg and Demeter, 1985), summarized in the scheme below (charged species active in luminescence are in bold). Charge pairs are stored as (i) the electron on the primary quinonic acceptor as Qa^- (centre closed) then on the secondary acceptor as Qb^- (centre open), (ii) four + holes are stored successively as S_{1+}, S_{2+}, S_{3+} and S_{4+} of the manganese oxygen-evolving complex, of which only the S_2 and S_3 states are able to recombine with Qa^- or Qb^-. The transient S_{4+} state goes back to S_0 while producing O_2 from two H_2O molecules. Luminescence-emitting pairs between positively charged (+) carriers on the donor side of PSII and electrons (–) on the acceptor side of PSII recombine via different pathways, part of them producing an excited chlorophyll centre P_{680}^*

$$\begin{array}{ccccccc} & & (+) & & (*) & & (-) \\ 2H_2O\rightarrow & (S_4/\mathbf{S_3}/\mathbf{S_2}/S_1S_0) & \rightarrow & \mathbf{Z^+}P_{680}\text{Pheo} & \rightarrow & \mathbf{Qa^-} & \mathbf{Qb^-}\rightarrow\text{Plastoquinones}\rightarrow\text{PSI} \\ & \downarrow O_2 & & & \mathbf{D^+} & & \end{array}$$

In a standard unstressed photosynthetic material (leaf, algae, cyanobacteria, intact chloroplasts, isolated thylakoids), a brief (5 μs) flash induces one charge separation per PSII centre which stabilizes as an S_2Qb^- pair, able to recombine with an emission of luminescence observable either as an exponential decay phase with $t_{\frac{1}{2}}$ ~40 s at 20 °C, or a so-called B band of thermoluminescence with a maximum temperature T_m between 30 °C and 40 °C. When sequences of 1–10 flashes are triggered before TL recording, the B band intensity oscillates with a period of 4 with maxima at two and six flashes, corresponding to the maximum S_2+S_3, the only two states able to yield luminescence upon recombination with Qa^- or Qb^-. The S_1 state is unable to recombine. Following a light period that creates an equipartition of S_0, S_1, S_2, and S_3, the states S_0 and S_1 remain stable during a dark adaptation whilst S_2 and S_3 are converted to S_1, resulting in a 1/4 S_0 3/4 S_1 distribution in dark-adapted material. In leaves, approximately 40% of Qb is reduced (Rutherford *et al*., 1984*a*), so that the Qb^-/Qb ratio weakly oscillates with a period of 2 according to flash number.

In isolated thylakoids at low pH (<6.5) the B band, induced by two flashes, splits into two bands B_1 (S_3Qb^-) and B_2 (S_2Qb^-), because S_3 is more destabilized by protonation than S_2 (Joliot and Joliot, 1980; Rutherford *et al*., 1984*b*; Demeter and Sallai, 1986; Miranda and Ducruet, 1995*b*). Upon treatment by a PSII-inhibiting herbicide (diuron, atrazine) which blocks the Qa to Qb electron transfer, the electron is stored as Qa^-, a less stabilized state (i.e. the activation barrier is smaller) than Qb^- and produces a Q^- band peaking at a lower temperature (around 5 °C at neutral pH) than the B band upon recombination with S_2/S_3. This band is associated with a C band at about 55 °C due to D^+Qa^- (Johnson *et al*., 1994), D^+ being the oxidized form of Tyrosine D on the inactive branch of PSII. The functional electron donor to the PSII P_{680} centre is Tyrosine Z: $Z+P_{680}^* \rightarrow Z^+ + P_{680}^-$.

A fast rereduction of Z^+ by S states occurs, except when the oxygen-evolving manganese complex is damaged, which leads to the emergence of A band Z^+Qb^- at about –15 °C. Other TL bands exist at lower temperatures which

are not fully explained and will not be considered here since they have been of little interest in plant stress physiology, at least up to now.

Twenty years ago, thermoluminescence brought a confirmation of the charge stabilization model of PSII. It remains a valuable technique that gives a global view complementing more analytical tools and it is particularly well adapted to the study of PSII mutants. Furthermore, like chlorophyll fluorescence, (thermo)luminescence is relevant to various levels of integration, from subchloroplast particles to algal cells or leaves (Farineau, 1993; Homann, 1999). However, applications to leaf photosynthesis have been relatively few until now, due to instrumental constraints and to a lack of understanding of *in vivo* signals. Several reviews already exist on photosynthesis TL (Sane and Rutherford, 1986; Vass and Inoue, 1992; Misra *et al*., 2001; Tyysjärvi and Vass, 2003). The present review will be focused on TL emission by leaves and will address the instrumental, theoretical and practical issues of measuring TL in leaf discs. Taking advantage of new and recently available components, simple instruments designed for leaf studies can now be built. The properties of TL bands already characterized in thylakoids or subchloroplast particles are modified *in vivo* by the cellular environment, particularly when leaves are kept unfrozen during the cooling step. Different types of stresses also modify the shape and intensity of the existing bands or enhance minor bands. Furthermore, a TL 'afterglow' band observable only in intact systems (intact chloroplasts, algae, leaf fragments) reflects the flow of electrons from reducing compounds present in the stroma to the plastoquinone pool and the quinonic acceptors of PSII. This back-transfer follows one of the pathways involved in the cyclic/chlororespiratory electron flow and appears to be a sensitive indicator of photosynthetic metabolism.

Although PSII is totally destroyed at about 60 °C, huge chlorophyll TL bands can be observed at higher temperatures. They correspond to a heat-enhanced chemiluminescence from molecular species generated by radical forms of oxygen, such as lipid peroxides, which accumulate in stress situations. Despite the fact that the mechanisms of high-temperature TL emission (HTL) is completely different from photosynthetic TL, recording through a single temperature scan from 0 °C to 160 °C on the same leaf disc both the photosynthesis TL bands and the oxidative stress HTL bands proves to be of practical interest in environmental physiology.

Instrument

Measuring thermoluminescence requires both the detection of a low far-red emission with the same maximum as prompt fluorescence at about 730 nm in leaves (another maximum at 685 nm is strongly reduced by chlorophyll reabsorption) and a flexible regulation of sample temperature allowing a fast cooling and a linear warming. This used to be achieved with costly, complex, cumbersome, and immovable apparatus, using liquid nitrogen for cooling (for example, see Ducruet and Miranda, 1992). New mass-produced (hence cheap) components, which appeared during the last ten years, have paved the way to affordable and easy-to-use instruments, easily transportable (as luggage) close to the experimental field. A truly portable instrument, powered by a 12 V battery and driven by a laptop computer is theoretically feasible, but TL experiments are done on dark-incubated material subsequently preilluminated under controlled conditions, so the interest in catching on-the-spot TL records from leaves submitted to natural sunlight has still to be documented. Leaf discs can be punched out in the field and dark-relaxed in a cooled box while being carried back to the TL instrument. Several TL instruments have been built in the Bioénergétique laboratory at CEA-Saclay as initially described in Miranda and Ducruet (1995*a*) and more completely in Ducruet *et al*. (1998). It might be and has indeed been reproduced or adapted in laboratories endowed with a mechanical workshop. (More information on instrument building and computer programs can be found in *Journal of Experimental Botany* online: see Supplementary data available in the online version of this article.)

Temperature regulation is performed with a 40×40 mm thermoelectric Peltier plate (Duratec from Marlow, or Thermatec from Melcor for measuring HTL up to 160 °C). A thin thermocouple is placed in the centre of the plate with thermal grease and covered by a copper or aluminium thin plate (1 mm) or adhesive sheet, which forms the bottom of a 25 mm hole bored in a heat-resistant plastic pressed against the plate through an O-ring. A drop of water (100 µl) is placed on the centre and the leaf disc is pressed on the bottom by a washer with a 20 mm inner diameter. A circular Pyrex window may be placed between the leaf and washer to reduce water loss from the sample during warming (except when recording HTL).

Luminescence emission from a leaf disc is sufficiently strong to be recorded with an analogue photomultiplier tube (PMT). Much more expensive photon counting systems are not necessary, their only advantage here being to allow the quantum noise to be known as the square root of counts, hence to assess rigorously the quality of fit (χ^2) of a simulated TL signal.. A compact red-sensitive PMT (Hamamatsu H5701-50 or H7711-50, with built-in amplifier and +15/–15 V power supply) is positioned 1.5 cm above the sample by a light-proof holder that can slide laterally to an illumination position where a light guide comes in front of the sample whilst the PMT is protected from strong actinic light (Ducruet *et al*., 1998; Fig. 1A). Alternatively, luminescence can be conveyed to the detector by a light guide, which prevents heating of the PMT windows during HTL measurements. In this new

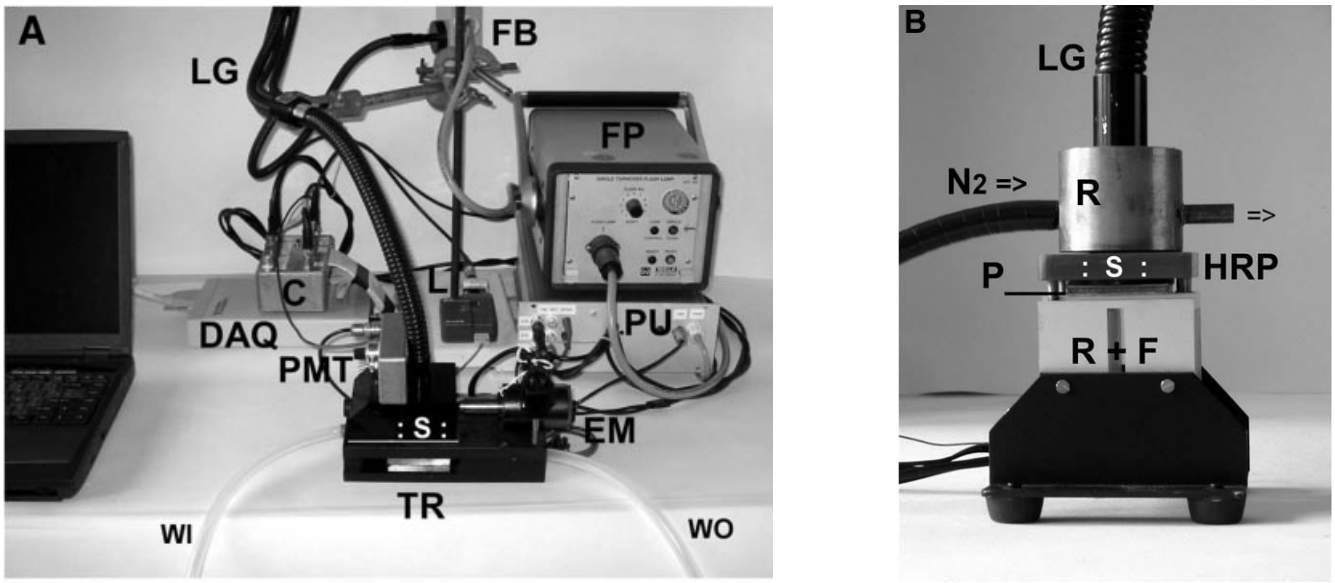

Fig. 1. Instrument for leaf thermoluminescence. (A) Version with detector (PMT) close to the sample and a water-cooled Peltier as described in Ducruet *et al.* (1998). The Hamamatsu photomultiplier module in its wiring box (PMT) and the common arm of a Walz light guide (LG) are held on a black plastic block sliding left to right on the temperature regulation block (TR), which contains the Peltier element pressed on a brass plate maintained at a constant temperature by a water flow (WI and WO: water inlet and outlet). In the present view, the sample location (S white, by transparency) is in front of the light guide which is used only for preillumination. For measurements, the PMT window is drawn towards the right on top of the sample by an electromagnet EM. The TL signal and temperature (L: thermocouple linearizer) are conveyed through the connection box (C) to two A/D ports of the interface National Instruments DAQ-Pad1200 (DAQ) linked to the computer (left) by the parallel port. DAQ+C also transmit from the computer the proportional temperature regulation and the PMT voltage commands through two D/A ports, the +5 V triggers of illumination and EM through five TTL ports. The power unit (PU) provides the variable current (0 to +5/–5 A) for temperature regulation, the +15/– 15 V for the PMT and +18 V pulse for EM. Walz single turn-over flash bulb (FB) and power unit (FP). (B) Sample holder of a new version with the PMT held apart on one arm of the light guide (not shown) and with an air-cooled Peltier. The Peltier plate P is pressed on the radiator with fan (R+F) by a heat-resistant plastic (HRP), in which a 25 mm diameter hole (white dots, by transparency) is bored. The sample (S) is placed on the bottom of the hole, against P (the same device as in above version, but hidden in A). The light guide (LG) is maintained always in front of the sample by a brass ring (R). Here, illumination and TL detection are done through LG. A gas (N_2) can be flushed on the sample: in HTL measurements, it both desiccates the leaf disc during warming and prevents any oxidation induced by high temperature. The PMT of version (A) can be used as the detector with an adaptor for fitting an arm of LG. All other parts (DAQ, C, L, PU) are identical.

configuration, the common part of a 4-arm or 5-arm PAM-Walz light guide is maintained on top of the sample by a holding ring (Fig. 1B). Luminescence is conveyed to the PMT via one arm of the guide whilst the four other arms are used for illumination (flashes, continuous visible or far-red light or weak blue LED for F_0 excitation) and, if needed, for recording fluorescence kinetics. The PMT is protected by a shutter during illumination periods. A loss of luminescence in the light guide is partly compensated by a higher luminescence collection at the guide entrance placed very close to the leaf disc. With an electric shutter, a whole preillumination/acquisition cycle, lasting about 10/15 min, can be programmed.

The instrument is driven by a PC computer, with a specially developed acquisition program, through an interface which should provide at least two analogue-to-digital (A/D) ports for temperature and TL signal entrance, respectively, a digital-to-analogue 0 to +5 V (A/D) port for proportional current regulation in the Peltier plate through a power amplifier, and five TTL ports providing +5 V to trigger various light sources and shutters. Either Advantech PCL718/818 cards plugged into a computer slot, or a National Instrument DAQ-Pad 1200 (Fig. 1A) on the computer parallel port are used. Before TL recording, it is possible to define up to 10 preincubation periods at different temperatures combined with a maximum of 10 flash sequences and/or 10 preillumination periods. An ultra-weak blue LED (480 nm) can be pulsed once or periodically to measure F_0 emission, subsequently separated from TL emission by TL interpolation.

Ultimately, TL measurement can be fully integrated in fluorescence and absorbance (e.g. P700) instruments such as PAM101, requiring basically a thermostated sample holder at the common part of a light guide, a PMT with shutter on one of the multiple arms and optionally a weak blue LED for F_0 excitation on another arm.

Signal analysis

Many minerals heated at hundreds of degrees emit luminescence, so that thermoluminescence has been used initially in geology, archeological dating and radiation dosimetry. The theory of charge recombina-

tion had first been worked out for minerals by Randall and Wilkins (1945). They considered that the recombination of fixed +/– charge pairs obeys a first order kinetics. This has been applied to the analysis of photosynthetic TL by Vass *et al*. (1981) using an analytical method. From the same theoretical background, a numerical method is also feasible (Ducruet and Miranda, 1992), the advantages of which are (i) to require no approximation, (ii) to take into account the actual temperature as measured under the leaf sample (hence the small irregularities on the simulation curve, reflecting those of the temperature gradient). It consists in calculating iteratively, from low to high temperature, the expected proportion of recombination $L(T)$ for each TL band at every measured temperature, using the Arrhenius–Eyring equation:

$$L(T)=N^{R}PT\Delta t \exp(-E_A/k_BT) \quad (1)$$

N represents the number of charge pairs still able to recombine at temperature T, and is decreased at each sampling step by $L(T)$. The initial concentration N_0 of identical charge pairs detected through the measuring system is the area of the resulting TL band. It can be known exactly with photon counting detection, but only as an arbitrary quantity with analogic detection (i.e. amplitude and integrated area of a TL band in arbitrary units). R is the order of reaction (generally R=1, as would be expected for charge pairs not exchangeable between PSII centres), P is the pre-exponential factor which can be related to the Arrhenius frequency factor s as $P=K(T)\times s$, $K(T)$ being an unknown factor; Δt is the duration of sampling step (generally 1 s); E_A is the activation energy, and k_B is the Boltzmann constant.

To what extent can an experimental TL signal be simulated using the theoretical equation (1)? Recombination of charge pairs in PSII is not exactly the same phenomenon as in minerals, since those charges are transferred downhill on carriers which are in temperature-dependent equilibrium. DeVault *et al*. (1983) and DeVault and Govindjee (1990) carried out a theoretical study of photosynthetic TL, showing that an apparent activation energy can be calculated even though several electron transfer steps have to be passed through from the stabilized state to the recombination step (for a recent review on the theory of photosynthetic TL, see Tyysjärvi and Vass, 2003). Practically, a first order theoretical simulation fits well to the central part of a B band (Table 1) induced by a single turn-over flash which only forms the pair S_2Qb^-, except at the low and high temperature edges due to the presence of minor bands. This shows that P and hence $K(T)$ are constant throughout a B band. By contrast, a S_2Qa^- Q band obtained in the presence of diuron is poorly fitted, because the recombination of the S_2Qa^- pair proceeds from a closed state Qa^- of all PSII centres to an open state Qa (Ducruet and Miranda, 1992). Indeed the F_0 fluorescence measured simultaneously drops, reflecting the progressive opening of centres. This raises the problem of retrapping: a quantum of excitation formed in the antenna by charge recombination migrates between chlorophylls almost as if it were coming from an absorbed photon, with a probability to be (re)trapped either by the same centre or by neighbouring centres. The retrapping is integrated in the $K(T)=P/s$ factor. In a B band, S_2Qb^- or S_3Qb^-, all centres are open (Qa) throughout the recombination process, so $K(T)$ remains constant, so that a good fit can be obtained. In a Q band, a closed S_2Qa^- centre opens as soon as charges recombine, but neighbouring centres change progressively from a closed to an open state as recombination proceeds, which increase the retrapping probability due to the connectivity of antennae between PSII units. Hence, $K(T)$ decreases as temperature increases. Attempts have been made to extract entropy value ΔS from the pre-exponential factor P and hence deduce the free energy of activation ΔE from the activation enthalpy $\Delta H=Ea$. However the frequency factor s is not experimentally accessible but only the pre-exponential factor $P=K(T)s$, where $K(T)$ is unknown.

Those theoretical considerations are important to clarify what can be reliably deduced from simulation. First simulation is necessary for the decomposition (it is not a deconvolution) of an experimental TL signal into elementary bands. This is done by adjusting the three parameters Ea, P and N_0 in equation 1 for each elementary TL band so that their sum fits the actual signal, by minimizing the sum of square of differences. A graphical fitting is first done interactively on the computer screen, with the amplified fitting residue optionally displayed, then refined by loop minimization. Simulation is theoretically justified, as explained above, for B bands, which are prominent in leaves. It provides an overall activation energy Ea (enthalpy) which reflects the band width (the higher Ea, the sharper the band) and allows quantitative comparisons to be done between the shape of similar bands in different samples. It also gives the relative intensity of each band. Finally, it allows the maximum temperature T_m of elementary bands to be known, which cannot be determined directly on the TL signal when bands strongly overlap, even less when they appear as shoulders.

Experimental

In the presently available TL instruments, pieces of leaves, usually discs, have to be excised just before the experiment and placed in the measuring chamber. In order to study the stabilization of charge pairs within PSII, it is possible to perform the dark-adaptation and the preillumination of the leaf sample in the same chamber. However, as TL becomes a tool in plant ecophysiology, it may become necessary to

Table 1. *Thermoluminescence bands observable in leaves* T_m values correspond to a 0.5 °C s^{-1} TL heating rate.

Name	T_m standard (~)	T_m shift	Origin	Comments
Photosystem II bands (after preillumination)				
A	–15 °C		Z^+Qb^- (S_3Qa^-)	Damage to Mn oxygen-evolving complex
Q	+5 °C		S_2Qa^-	Damage to secondary Qb quinonic acceptor (induced by diuron or atrazine-like herbicides)
B	+35 °C	+28 °C	S_2Qb^-	Typically observed after one flash (Fig. 3A, B)
		+22 °C	S_3Qb^-	Typically observed after three flashes (Fig. 3B)
AG	+45 °C	+35 °C	S_2/S_3Qb+e^-	Typically induced by 720 nm light (sometimes by flashes)
C	+55 °C		D^+Qa^-	Appears when Qb is blocked by diuron or damaged (D is the non-functional donor to PSII, Z is the functional one)
Oxidative bands (not dependent on preillumination)				
HTL1	65–85 °C		Aldehydes+H_2O_2?	Also pseudo-HTL2 in wet samples (Fig. 4)
HTL2	120–140 °C		Lipid peroxides	Correlated with content in lipid peroxides in dry samples (Fig. 4)
HTL3	> 160°C		Induce by warming	Increased by O_2, reduced by N_2 flushed during TL warming

submit the attached leaf to more elaborate conditioning outside the TL chamber, for example, in a gas exchange clip, then quickly to punch out a disc for TL recording. .

Historically, charge separation before TL had to be induced by continuous illumination at low temperatures or even throughout a slow (for instrumental reasons) cooling process. This had allowed some major TL bands to be detected in the 1970s, but failed to reach mechanistic assignments because complex signals thus generated were resulting in undefined charge patterns, with possible artefacts due to photoinhibition. Short sequences of single turn-over xenon flashes, each of them inducing one and only one charge separation per PSII centre, provided a unique tool to create well-defined charge patterns on PSII electron carriers and therefore played a major role in the identification of TL bands in the early 1980s (Rutherford *et al*., 1982, 1984). Excitation by short flash sequence (at least one and two flashes and a dark control) should always be done to characterize control and treated leaf samples, notwithstanding further experiments using continuous illumination.

Freezing or not freezing? In conventional TL protocols, the sample is deep-frozen possibly down to liquid nitrogen temperature to be sure that no recombination occurs before TL recording. In some plant species such as grapevine, freezing produces a complete change of the TL signal with sharp peaks near 10 °C of unknown origin (Ducruet *et al*., 1998; Homann, 1999). This might be due to the release, through frost-disrupted membranes, of active compounds stored in the vacuole such as phenolics, in lesser amounts in spinach or pea leaves. A complete study on the effect of freezing on TL emission has been carried out by Homann (1999). As a consequence, a comparison should be done for every new leaf material between TL emission in frozen and unfrozen samples, to determine if freezing distorts the signal. If not, it remains, as further discussed below, that freezing acts as an uncoupler and can be used as a tool to relax the dark-stable proton gradients. It should be noticed that the TL signal, even undistorted, has a lower intensity after freezing (non-radiative recombination may occur even at low temperatures by tunneling, i.e. temperature independent non-radiative recombination).

This carries instrumental consequences: Peltier plates provide both a rapid cooling (20 °C to 0 °C in a few seconds using 5 A current) and a better control of the lower temperature than by dipping the holder in liquid nitrogen. Furthermore, a single-stage Peltier plate with a water flow at 0 °C can reach –30 °C. This allows the resolution of the A band at –15 °C. When freezing is avoided, the TL gradient should be started at 0 °C immediately after preillumination in order not to let the Q band, peaking near 5 °C, to be partly discharged. This Q band appears as a shoulder at the beginning of the thermogram whereas the A band is not seen and the major bands B1, B2, AG, and C can be fully resolved (Table 1).

Various treatments or stress factors may affect TL emission, compared to a control, by inducing new bands,

by modifying the shape and/or the intensity of existing bands (essentially the B band). Comparing the absolute intensity of a TL band between different samples raises the same problem as using the absolute values of fluorescence parameters such as F_0 or F_m, i.e. the variability of these measurements due to natural heterogeneity between leaf samples. This variability can be reduced by comparing leaves of the same age and development. However, it is also advisable to relate those values to an internal standard, either another TL band when possible or the F_0 level measured simultaneously by a pulse of ultra-weak blue light.

Figure 2 show an example of TL and fluorescence F_0 recorded at two heating rates after a far-red excitation. The TL signal obtained at 0.5 °C s^{-1} show a peak at 42 °C and a shoulder near 20 °C, which correspond to an afterglow (AG) band and a downshifted B band, respectively, as explained below. A slower heating rate (0.25 °C s^{-1}) results in a downshift of the bands, since more recombination events occur in the same temperature interval (all T_m in this review are at 0.5 °C s^{-1}). The F_0 is constant up to 30 °C and can be used as an internal standard to calibrate the intensities of TL bands. It starts increasing at about 40 °C, sometimes with a shoulder in the 30–40 °C range, and peaks above 50 °C (Schreiber and Berry, 1977), reflecting heat damage to PSII.

Classical TL bands *in vivo*

The B band at about 35 °C is prominent in a healthy dark-adapted leaf submitted to one or several single turn-over flashes, although minor bands can also be detected: the Q band on the lower edge, AG or C bands on the upper edge. Increase of the Q band (T_m ~5 °C), generally associated with a C band (~55 °C), reflects damage to the secondary quinonic acceptor Qb of PSII, induced for example by photoinhibition (Janda *et al*., 1992). The A band (~ –15 °C) appears in the case of damage to the oxygen evolving complex. However, continuous illumination at freezing temperatures can induce these Q and A bands as artefacts, another reason for preferring flash excitation. For routine work, two flashes can be used since they generate S_2 and S_3 states from the dark-stable S_0 and S_1, hence the strongest B band.

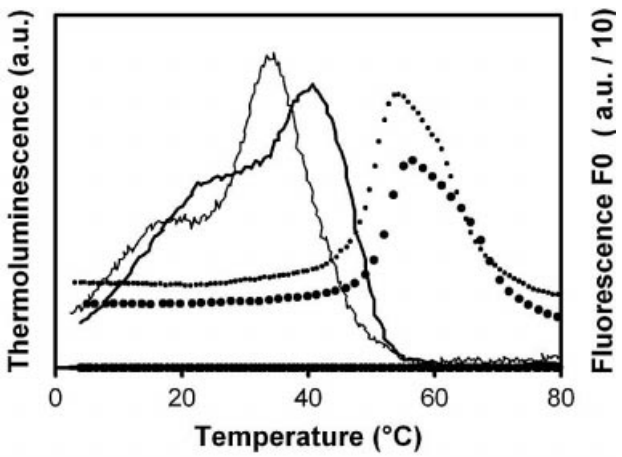

Fig. 2. Thermoluminescence (lines) and fluorescence F_0 (dots) from of leaf disc of maize inbred line after 30 s far-red light excitation. Heating rates: 0.5 °C s^{-1} (thick) and 0.25 °C s^{-1} (thin). F_0 is excited by a weak 480 nm LED pulsed every 3 sampling steps and separated from the overall signal by subtracting the value of interpolated TL signal (amplitude divided by 10 on graph).

Besides the emergence of new stress-induced TL bands, the characterization of the B band(s) itself can be very informative. In frozen spinach leaves, the B band exhibits the classical period 4 oscillation pattern (Rutherford *et al*., 1984*a*). During photoinhibitory treatments, the intensity of the B band decreases in parallel to the F_v/F_m fluorescence ratio, reflecting the destruction of PSII centres. After cold adaptation, the activation energy is decreased in spinach leaves (Briantais *et al*. 1992) and in some chilling-tolerant inbred lines of maize (unpublished results). A slightly lower activation energy *Ea* could be observed in crop plant species adapted to cool climates compared to thermophilic species, and their *Ea* was further decreased upon cold hardening (Glémin *et al*., 1992). Such experiments were done after freezing the leaf fragments to –40 °C, which breaks the thylakoid membrane so that PSII centres are in a fully relaxed state, without an influence of a dark-stable transthylakoidal proton gradient.

Compared with frozen leaves in which the B band peaks at about 35 °C, whatever the flash number, unfrozen leaves show a B_2 S_2Qb^- band after one flash near 32 °C and a B_1 S_3Qb^- band after three flashes near 28 °C. After two flashes, B_2 is prominent with B_1 appearing as a shoulder on its upper edge (this B_1 B_2 nomenclature can be confusing, and is indeed confused in the literature, since historically numbering has been done from low to high temperature, and it was only known later that B_1 is induced by two flashes and B_2 by one flash). These downshifts can be explained by an acidic pH of the lumen, whereas the stroma pH remains fairly constant (Heldt *et al*., 1973), which destabilizes S_3 strongly and S_2 to a lesser extent (Joliot and Joliot, 1980; Rutherford *et al*., 1984*b*; Demeter and Sallai, 1986; Miranda and Ducruet, 1995*b*), as later explained by the pH-dependent protonation pattern of S states (Lavergne and Jünge, 1993).

Figure 3 shows examples of B bands produced by flash excitation on healthy leaves. In Fig. 1A, the S_2Qb^- charge pairs generated in a maize leaf by a single turn-over flash produce a TL band that can be satisfactorily fitted by a one-component numerical simulation using equation (1) as previously explained. The apparent activation energy *Ea*=1.04 eV corresponds to that of a thermophilic species, whereas *Ea* found in cold-tolerant species are somewhat lower, between 0.75 and 0.9 eV (Briantais *et al*., 1992; Glémin *et al*., 1992). In most cases, the fit is not so good due to the presence of a small Q band on the lower edge

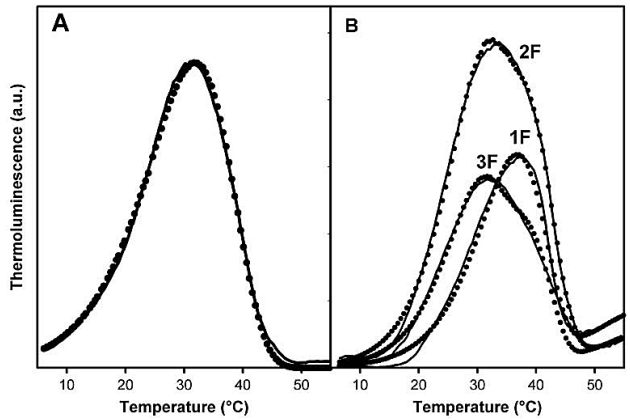

Fig. 3. B bands generated by single turn-over flashes. Lines: experimental signal. Dots: simulation. (A) B band generated by one flash in a leaf disc of a maize inbred line. (B) B bands generated by one, two or three flashes in pea leaflets.

and an AG band on the upper edge of the B band. Figure 3B shows a typical example of B bands generated by flashes in pea leaves. It should be noticed that TL intensities between different leaf samples (here pea leaflets) cannot be exactly compared. Nevertheless, the B band is clearly maximum after two flashes (all centres are in the S_2 or S_3 states) then decreases after three flashes. A B band much higher after three flashes than after one flash would indicate that the flash is not saturating, thus inducing 'misses' (i.e. a fraction of centres does not undergo charge separation, due to the scarcity of absorbed photons). The B band after one flash (S_2Qb^-) can be simulated with one component (another component a higher temperature was introduced to correct the baseline). The simulation is not so simple after three flashes (S_2Qb^-), since S_2Qb^- (due to misses) and AG (explained below) components had to be introduced. Finally, The B band after two flashes was fitted with the three components, with *Ea* and *P* for S_2Qb^- and S_2Qb^- blocked. Although a minimum number of three components is used here, fairly good fits (local minima) can be obtained with completely different sets of parameters: a decomposition led only by the search for an absolute minimum can be misleading, because the fit may be biased by the presence of minor bands Therefore, decomposition of complex signals should be started with components determined on simpler signals. Simulation should be considered mainly as a practical tool for a signal decomposition, taking into account the theoretical charge patterns. It is not always necessary: in Fig. 3B, the B band after two flashes is complex, but the apparent T_m after one and three flashes provide a good estimate of the downshift due to S_3 compared to S_2, ascribable to an acidic lumen pH.

Afterglow (AG)

Bertsch and Azzi (1965) have described a luminescence bounce superimposed on luminescence decays following a far-red (>700 nm) illumination. Björn (1971) showed that it was related to cyclic electron flow and provided evidence of the enhancing effect of temperature on this 'afterglow'. This emission reflects a back-flow of electrons from reductants present in the stroma to the quinonic acceptors of PSII, allowing their recombination with S_2 and S_3 states (Sundblad *et al*., 1986, 1988). Whilst S_2Qb^- and S_3Qb^- centres produce a B band, the S_2Qb and S_3Qb centres should not lead to luminescence emission, unless an electron is progressively fed back to Qb, resulting in an AG emission. It should be stressed that this emission, even though generally induced by far-red light which preferentially excites PSI, is originating from PSII, as showed by its period-4 oscillation and by luminescence emission spectroscopy (Hideg *et al*., 1991). The weak absorption of far-red light by PSII is sufficient to create luminescence active states S_2 and S_3. This AG emission initially reported in algae also occurs in higher plants (Nakamoto *et al*., 1988).

The afterglow emission is enhanced and accelerated by temperature elevation up to 40/45 °C (Björn, 1971), the threshold for heat damage to PSII (Schreiber and Berry, 1977). However, more subtle changes occur in the 25–40 °C temperature range. PSII centres are located mainly in grana and PSI centres in stroma lamellae. Moderate heating above 30 °C induces an unstacking (Weis, 1984; Sundby *et al*., 1986) accompanied by structural changes (Thomas *et al*., 1986). Photoacoustic spectroscopy confirmed a triggering of PSI cyclic electron flow in this temperature range, driving electrons from stroma reductants towards the acceptor side of PSII (Havaux, 1996), consistent with the role of state transitions in the induction of the cyclic pathway (Finazzi *et al*., 2002). In order to avoid triggering the AG process during the far-red excitation period, a temperature jump method can be used, which consists in illuminating the sample at 10 °C, then quickly raising the temperature to 25 °C, 30 °C or 35 °C. An alternative method is to warm the sample progressively after far-red excitation at 10 °C or 0 °C, in order to reveal the temperature-induced back-transfer of electrons towards PSII as a sharp TL band at about 45 °C (Miranda and Ducruet, 1995*a*; Fig. 2). The sharp band at 45 °C exhibits the properties of an afterglow emission, with a maximum AG/B band intensity ratio after three flashes and a similar sensitivity to characterizing chemicals. Addition of an uncoupler or freezing below –5 °C suppresses the AG bands and the downshift of the B band, resulting in a unique band at 35 °C. A basic property of the AG emission is its suppression by 5 μM antimycin A, as already shown by Björn (1971), a low concentration which also selectively inhibits the ferredoxin-plastoquinone-reductase or FQR pathway, but not the NAD(P)H-plastoquinone-oxidoreductase or NDH pathway (Ravenel *et al*., 1994; Bendall and Mannasse, 1995; Scheller, 1996). Consistently, the AG emission remains almost unchanged in NDH-deficient mutants of tobacco (L Cournac and J-M

Ducruet, unpublished data; A Liszkay-Krieger, personal communication). The molecular background of the FQR activity remains unknown.

In healthy leaf material a sharp AG band reveals the induction by heat of the corresponding cyclic pathway. A broadening and/or a downshift of the AG band suggest that this cyclic electron flow is already active at lower temperatures, so that the effect of heat induction is less pronounced. Cyclic electron flow seems to be triggered by stress situations in order to meet an increased demand in ATP (Manuel *et al*., 1999). Under illumination, the AG band is downshifted in some plant species (see Fig. 3) and it resumes its location at 45 °C within a few hours in the dark, reflecting the activation of a cyclic pathway by light and its progressive inactivation in the dark (M Roman *et al.*, unpublished data). Reduction of plastoquinone in the dark is also part of the chlororespiratory pathway discovered in *C. reinhardtii* (Bennoun, 1982) and in higher plants (Garab *et al*., 1989), the physiological role of which is still unknown.

In some particular conditions, an AG emission appears after white light or xenon flash illumination. This occurs when the use of photosynthetic energy (ATP+NADPH) is slowed down by a lack of CO_2 or phosphorus (Mellvig and Tillberg, 1986; Palmqvist *et al*., 1986). It can be observed in young pea leaves (Miranda and Ducruet, 1995*a*). In a CAM-inducible species, an AG TL band appears when CAM metabolism is activated, in correlation with the increase in the dihydroacetone-P/phosphoglyceric acid ratio (Krieger *et al*., 1998), an indicator of the assimilatory potential [NADPH+ATP] (Heber *et al*., 1986). The AG band induced by two flashes also reflects the chloroplast energetic state in pea leaves (Roman and Ducruet, 2000).

The afterglow is both a time-dependent and a temperature-dependent process, and hence not relevant to the Randall–Wilkins model of pre-existing charge pairs. Indeed, in some cases, no TL simulation could be done. However, a satisfactory fit is generally possible, suggesting that the temperature and not the back-transfer time is the limiting factor in TL afterglow emission (e.g. TL signals in Fig. 2 can be decomposed in the two bands B and AG, which have not been shown for clarity).

In agreement with several authors who reported various stress effects on the far-red induced afterglow observed on luminescence decays (Mellvig and Tillberg, 1986; Schmidt and Senger, 1987), the far-red-induced AG band of TL appears as a sensitive stress indicator (Janda *et al*., 1999, 2000). Perhaps too sensitive? Indeed, it is modified by external factors such as previous light episodes, morning or evening sampling, sunny or cloudy day, temperature, and age of leaves (Roman, 2002), all factors which leave the fluorescence parameter F_v/F_m unchanged (but may affect the q_P and q_N quenchings). More work is needed to harness the AG emission into a reliable tool for ecophysiology.

High temperature bands

When a sample is submitted to stress treatments, strong TL bands can be observed above 50/60 °C without prior illumination, in a temperature range fully destructive for PSII. They constitute the chemiluminescence high-temperature bands or HTL, unrelated to photosynthesis with the exception that dark-excited chlorophylls are luminescence emitters in the red.

Around 1990, scientists at the university of Moscow reported TL bands above 60 °C, with a main band at 130 °C in algae or leaves submitted to oxidative stress (Venediktov *et al*., 1989; Vavilin *et al*., 1991; Merzlyak *et al*., 1992). Independently, a band peaking at 75 °C induced by oxidative treatments was described by Hideg and Vass (1993). A similar band was found in tobacco leaves treated by a fungal elicitor (Stallaert *et al*., 1995) and in greening barley leaves (Marder *et al*., 1998), without bands at higher temperatures. Finally, the 130 °C band reappeared when the sample was pressed uncovered in the dry atmosphere of a 'classical' TL set-up where liquid nitrogen acted as a drying vapour trap, the intensity of this band being correlated with the content of lipid peroxides (Vavilin and Ducruet, 1998). By contrast, when samples (chloroplast, algae, leaf squares) were kept in water medium up to 100 °C to prevent desiccation, as usually done in TL photosynthetic studies, only the band at about 75 °C could be seen (Fig. 4). The 130 °C band in an uncovered leaf sample was not reduced by flushing the compartment with nitrogen gas, demonstrating that peroxides were not formed during warming, but disappeared when the sample was maintained wet by a window,

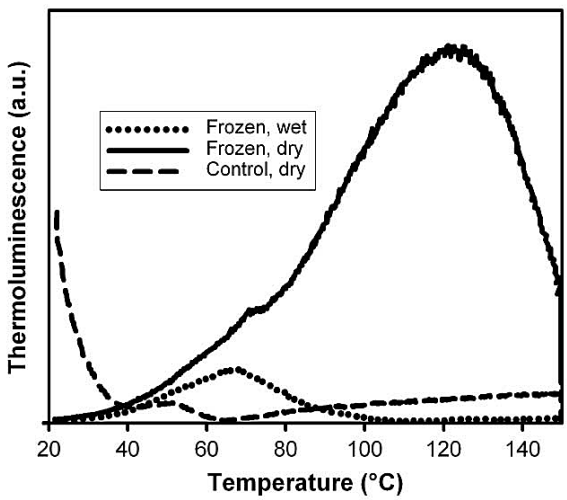

Fig. 4. HTL emission from spinach leaf discs stuck on a 24 mm diameter copper sheet, frozen for 20 h at –20 °C then thawed for 1 h at room temperature. Dry: disc was desiccated under vacuum before TL recording without glass window. Wet: undesiccated disc, TL recording with glass window above sample. Control: unfrozen, dry conditions.

suggesting that the lipid peroxides were hydrolysed during the heating process before radiative thermolysis could occur (Ducruet and Vavilin, 1999). So, the 75 °C TL band recorded in wet conditions would be a pseudo-band resulting from the competition between radiative thermolysis (rising edge) and heat-enhanced non-radiative hydrolysis (falling edge)

Other bands have been reported in the 60–90 °C temperature range. In barley cultivated in low light, a band near 60 °C was observed without prior illumination (Skotnica *et al*., 1999). In crucifers such as *Arabidopsis thaliana* (Havaux and Niyogi, 1999) or rapeseed, a sharp band at 70 °C, also present in unstressed controls, might be tentatively ascribed to the chemiluminescence of compounds specific to crucifers. The addition of both aldehydes and H_2O_2 to chloroplast suspensions produced TL bands near 60/70 °C (Ducruet and Vavilin, 1999).

Practically, the 130 °C can be used as an indicator of oxidative stress (Havaux and Niyogi, 1999). Care should be taken not to cover the sample with a window, and to let it dry during TL warming. Flushing nitrogen gas in the holder during measurements can speed up drying and make sure that no sample oxidation occurs (although this has not been observed in air, except at temperatures above 140 °C). A problem is the wilting or popping up of leaf sample above 100 °C due to dryness. This can be avoided either by a metal grid pressing on the leaf or by sticking the leaf disc on a piece of aluminium or copper adhesive sheet for electronics. In this latter case, storing leaf discs stuck on a thin flat metal sheet in a cool (but not freezing) dry oxygen-free vessel and sending these for measurements could be considered. Another potential application of HTL emission would be the imaging of oxidative stress in whole leaves.

Perspectives

TL is clearly a destructive technique although to some degree non-invasive, since it preserves the leaf integrity until the measuring step, somewhat like a biochemical analysis done on a freshly excised sample. Warming a spot of an attached leaf would be feasible using a variable temperature air flow or an infrared heater, but lateral diffusion of daylight through the mesophyll would imply that it works in full darkness. Fresh leaf discs offer a level of integrity even better than intact chloroplasts or protoplasts, and are considered as intact systems for studying photosynthetic metabolism. Ultimately, an outdoor light-tight TL measuring head for punching out a disc from a leaf under sunlight and immediately starting the gradient up to 160 °C is technically feasible: this would open an entirely new field of research.

Luminescence is the informative phenomenon and TL is a particular technique providing a better resolution of emitting charge pairs at the expense of a thermal treatment applied to the sample. Among alternative methods, the so-called 'delayed light emission', not to be confused with delayed fluorescence or delayed luminescence (perhaps light-modulated luminescence would be a better term) consists of a pulsed illumination with recording of fluorescence induction through the light pulses and of luminescence induction through the dark intervals. This luminescence kinetics reflects the energization of chloroplasts up to a stationary state, which cannot be done in one TL scan. DLE has produced intriguing results such as temperature breakpoints consistent with the thermal adaptation of various plant species or differences in the characteristic freezing temperatures which uncouples thylakoids in wheat varieties of contrasting frost hardiness (Fedoulov, 1998), although the intertwining of excitation and detection makes interpretation more complex (Malkin *et al*., 1994). DLE deserves to be reappreciated as a tool complementary to TL, with basically the same instrument, using LED pulses for excitation and a modulated shutter or attenuator protecting the PMT during the light pulses.

In conclusion, the progress in instrumentation and in understanding TL signals *in vivo* allows the use of (thermo)luminescence in plant ecophysiology in order to complement gas exchange, chlorophyll fluorescence, and leaf absorption/reflectance measurements. Several applications to investigate abiotic and even biotic (Rahoutei *et al*., 1999) stress have already been published. The potential of photosynthetic TL and of HTL has now to be assessed within the frame of ecophysiological research.

Supplementary data

More information on instrument building and computer programs can be found at *Journal of Experimental Botany* online.

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