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Lipid peroxidation in tobacco leaves treated with the elicitor cryptogein: evaluation by high-temperature thermoluminescence emission and chlorophyll fluorescence

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Abstract

Treatment of excised tobacco leaves with the fungal elicitor cryptogein progressively induced lipid peroxidation. In a first step, evidence was provided by the accumulation of thiobarbituric acid reactive substances (TBARS) and in a second step, the process was monitored for a 26 h period by high-temperature thermoluminescence (TL) emission, showing a close correlationship with the TBARS data. Differences in the temperature-associated F_0 rise (constant fluorescence) and in fluorescence emission spectra point to a progressive destabilization of the thylakoid membrane, especially affecting Photosystem II (PS II). In parallel, the PS II quantum efficiency ($\Delta F/F_m$) and the F_v/F_m ratio of chlorophyll fluorescence induction decreased significantly over the 24 h period.

Keywords: Hypersensitive response; Lipid peroxidation; Thermoluminescence; Fluorescence; (*N. tabacum*)

1. Introduction

The defense responses of plants against incompatible pathogens consist of a diverse array of metabolic alterations eventually leading to necrotization of cells and limitation of parasite invasion. Early events in the hypersensitive reaction of cells include membrane depolarization [1–3], net efflux of electrolytes [4] and activation of a plasma membrane redox system generating active oxygen species [5,6]. In addition, evidence is accumulating pointing to an involvement of lipid peroxidation as an important functional step in the overall response, both upon treatment with bacterial [7–9] and fungal [10–15] elicitors. The peroxidative breakdown of lipids results in a formation of hydroperoxides which are converted to a range of secondary products such as active oxygen species, lipid radicals, aldehydes, alkanes, ketols, oxo-acids and (methyl)-jasmonic acids [16]. Oxygen and lipid free radicals damage the membrane structure and organisation as well as proteins and amino acids [16]. Other breakdown products have been implicated in triggering defense gene expression ((methyl)-jasmonic acid) [17] and anti-pathogenic action (aldehydes, jasmonic acid) [9]. In the literature, no consistency exists regarding the inducing agents of lipid peroxidation in plant-pathogen interactions. Arguments can be found both for an enzymatic initiation by lipoxygenase [9,12–14] and for a non-enzymatic initiation by active oxygen species [7,8,11,15,10]. Anyhow, the general mechanism of lipid peroxidation involves an interaction between oxygen containing radicals leading to conversion of organic molecules and oxygen into their free radical and excited forms. Some of these excited forms are capable of transfering excitation energy to chlorophyll [18]. From this viewpoint, a study of thylakoid lipid peroxidation and its effect on the photosynthetic capacity would provide useful information. A valuable tool in this study is the investigation of thermoluminescence emission at high temperatures. Recently, two groups have reported high-temperature thermoluminescence (TL) bands which does not result from a

Abbreviations: A.U., arbitrary units; BHT, butylhydroxytoluene; F_0, constant fluorescence (reaction centers open); F_m, maximal fluorescence (reaction centers closed); F'_m, maximal fluorescence at any time of the induction curve; F_v, variable fluorescence ($F_m - F_0$); ΔF, $F'_m - F$; MDA, malondialdehyde; PAM, pulse amplitude modulation; TBARS, thiobarbituric acid reactive substances; T_c, critical temperature; TCA, trichloroacetic acid; TL, thermoluminescence.

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charge recombination of Photosystem II redox components but probably from an energy transfer of lipid peroxidation products towards chlorophyll [19,20]. The inconsistency of the reported emission temperatures of this TL band (120° C and 75° C, respectively) is likely to be linked to the difference in experimental materials and in heating rates. The intensity of the high-temperature peak was correlated with the concentration of malondialdehyde (MDA) which is a product of lipid peroxidation [21].

For the past few years, our group has been interested in the multiple responses of tobacco (*Nicotiana tabacum*) upon treatment with a proteinaceous elicitor, cryptogein, a polypeptide produced by *Phytophthora cryptogea*. When added to tobacco cell suspensions, it rapidly induced an alkalinization of the extracellular medium, a leakage of potassium [22] and a transient production of active oxygen species within 60 min after treatment [23]. Later on, ethylene and phytoalexins accumulated after a lag-phase of 1 and 6 h, respectively [24]. When applied to the petiole of leaves, cryptogein similarly enhanced ethylene and phytoalexin production resulting in macroscopically observable HR-like necrosis. Microscopical observations of the chloroplasts revealed a progressive unstacking of the grana in the cryptogein-treated leaves [25]. Taken together, these observations led us to suspect a possible role of lipid peroxidation in the overall process of the tobacco-cryptogein interaction.

In the present study, we were able to approach the induction of lipid peroxidation in plant-fungus interactions from a particular point of view. Within the model investigated, namely the interaction of tobacco leaves with cryptogein, we combined thermoluminescence and fluorescence data both to estimate the degree of peroxidation and to reveal accompanying alterations in the photosynthetic apparatus.

2. Materials and methods

Tobacco (*Nicotiana tabacum* var. *Xanthi*) plants were grown in a greenhouse for 9 weeks. Leaves of 2 to 4 g were selected and treated with cryptogein, a fungal elicitor purified from *Phytophthora cryptogea* according to Ricci et al. [26]. A 10 μl drop of an aqueous solution containing 2.5 μg of cryptogein was put on the cut surface of the excised leaf. Control leaves were treated with 10 μl of water instead of cryptogein solution. Full absorption was ensured by triple rinsing with 10 μl of distilled water. Leaf petioles were placed in water and kept in the dark at room temperature for periods of 0 to 24 h. Incubations were performed at 22 to 24° C.

Thermoluminescence (TL) measurements and signal analysis were performed according to Ducruet and Miranda [27]. Briefly, a piece of leaf was inserted into the cuvette of the sample holder of a laboratory-made set-up. The sample was cooled down to 0° C and eventually illuminated by single-turn-over flashes. Next, the sample was heated to 100° C at a rate of 30° C/min. TL was recorded at wavelengths above 670 nm by a cooled photomultiplier, linked to a photon counting system.

Constant fluorescence (F_0), periodically excited by an ultra-weak 480 nm light, and thermoluminescence were also simultaneously recorded [27] at a slow heating-rate (3 C° /min) from 0 to 100° C.

Fluorescence emission spectra at 77 K were obtained according to Weis [28], by grinding in liquid nitrogen a 0.25 cm^2 piece of leaf in the presence of 0.5 ml water and 1.5 ml quartz particles. Final chlorophyll concentration was approximately 2 μg/ml. Fluorescence was excited by a 634 nm Helium-Neon laser. Monochromator scanning (1 nm optical bandwidth) and recording were driven by a home-made computer program.

The whole induction kinetics was followed with a modulated PAM fluorimeter (Walz, Germany), under a continuous illumination (634 nm, 135 $\mu E\, m^{-2}\, s^{-1}$) and 5000 $\mu E\, m^{-2}\, s^{-1}$, 1 s light pulses [29].

Lipid peroxidation was estimated from the accumulation of thiobarbituric acid-reactive substances (TBARS). Leaves were ground in the presence of liquid nitrogen for 40 to 80 s according to their fresh weight. Ice-cold 0.2 M sodium-phosphate buffer (2.5 ml buffer per g fresh weight) containing 1% Triton X-100 and 0.01% BHT was added. The extract was centrifuged for 20 min at $10\,000 \times g$ and the pellet was discarded. A 150 μl aliquot of the leaf extract was mixed with 300 μl TCA (10%) and 450 μl TBA (0.67%) and heated in a boiling water bath for 15 min. After cooling, the absorbance at 532 nm was determined and corrected for non-specific dissipation at 600 nm. The concentration of lipid peroxides was estimated as the amount of malondialdehyde (Extinction coefficient = $156 \cdot 10^3\ M^{-1}\, cm^{-1}$.)

Data were analysed using unpaired *t*-tests and significance assigned if $P \leq 0.05$. Pearson's product moment-correlation coefficients (c.c.) were calculated to measure linear association.

3. Results

A high-temperature TL band was detected both in dark-adapted and flash-illuminated leaf fragments, to a much greater extent in cryptogein-treated samples than in control leaves. Its maximum temperature was dependent on the heating-rate and was located near 90° C for 30° C/min (Fig. 1) and 70° C for 3 C° /min (Fig. 2) temperature gradients. The peak area in leaves treated with cryptogein increased drastically during the 26 h period after treatment (Fig. 3). No such rise was observed for control leaves. The variability between the peak area of different leaves submitted to an identical treatment is likely to be due to a heterogeneous dispersion of the cryptogein molecule, leaf size, leaf age, and also to varia-

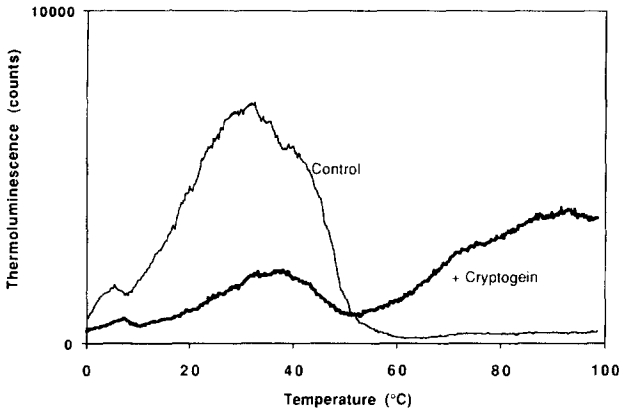

Fig. 1. Thermoluminescence emission of tobacco leaves after a one flash illumination as a function of temperature. Leaves were treated with a cryptogein solution (thick line) or with H_2O = control (thin line) and kept in the dark for 17 h. The flash was given at 0° C followed by heating at a rate of 30 C°/min.

tions of optical properties between leaf samples. Consistent results were obtained when comparing several leaves of similar sizes incubated simultaneously. Since treatment with cryptogein induces a fresh weight loss of the leaves, untreated leaves were subjected to a desiccation comparable to that of cryptogein-treated leaves in order to be sure that the increase of the high-temperature band was not due to the dehydration phenomenon.

Upon illumination with one to three flashes at 0° C, at a heating-rate of 30 C°/min, another thermoluminescence band, the well-characterized B band, was observed next to the 90° C TL band. The peak arising after one flash showed a maximum temperature, T_m, at about 32° C, corresponding to the recombination between the electron of the secondary electron acceptor Q_B^- and a positive charge on the S_2 state of the water oxidizing complex [30]. This T_m remained almost unchanged during the 26 h experiment, in both treated and control samples. Three flashes predominantly led to the $S_3Q_B^-$ recombination state, which was differentiated from the $S_2Q_B^-$ component by signal decomposition. The $S_3Q_B^-$ band peaked at 27° C at the beginning of treatment, and its T_m slightly decreased, more in treated than in control samples (towards 15° C and 21° C resp., before). In both cases (one and three flashes), the area of the B band of treated samples started decreasing 12 h after cryptogein treatment and vanished completely towards 24 h after treatment. It should be noted that a shoulder appears near 42° C in unfrozen leaves (Fig. 1), corresponding to the luminescence afterglow first reported by Bertsch and Azzi [31], for which a detailed study will be published elsewhere.

The evolution of thiobarbituric acid reactive substances (TBARS) is shown in Fig. 4. TBARS increased about 3-fold in cryptogein-treated samples whereas their amount remained stable in control leaves. These TBARS data showed a positive correlation (c.c. = 0.951) with the TL results.

The constant fluorescence F_0 versus the temperature (Fig. 2), recorded at a slow heating-rate (3 C°/min), showed a peak at 52° C in both dark-adapted control and treated leaves. The maximal intensity of this peak, which is where F_0 and F_m converge [32], related to F_0 below 20° C, progressively decreased in cryptogein-treated samples down to 2.7 ± 0.4 compared to 4.5 ± 0.1 in control samples, as averaged from four independent experiments after a 24 h incubation. The critical temperature, T_c, corresponding to the beginning of the F_0 rise, was 45.7 ± 0.2° C in control samples. In the first 14 h of cryptogein incubation, T_c decreased to 38° C, afterwards it could not be determined anymore. It should be noted that the F_0 shoulder observed near 35° C represents a distinct phenomenon and was discarded in the analysis of F_0.

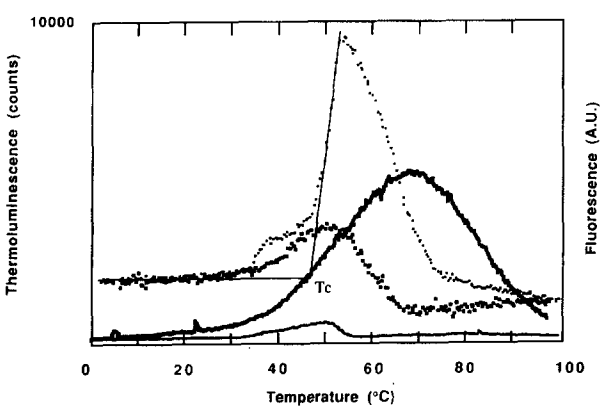

Fig. 2. Representative examples of thermoluminescence (lines) and F_0 (dots) emission in tobacco leaves without preillumination. Samples were heated at a rate of 3 C°/min. T_c = critical temperature. Leaves were treated with a cryptogein solution (thick line and dots) or with H_2O = control (thin line and dots) and kept in the dark for 24 h at room temperature. Note: TL emission below 45° C can be ascribed to recombination of charges stable in the dark, such as Q_B^- and D^+ [27]. The photon counting efficiency was 0.4-times that of Fig. 1.

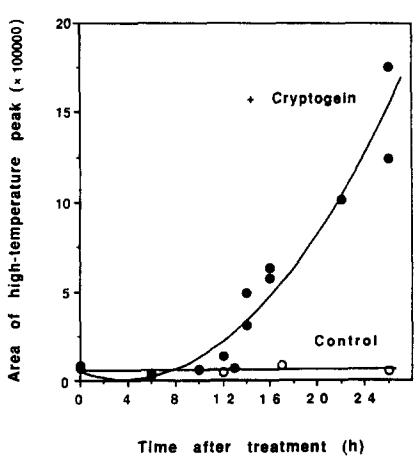

Fig. 3. Signal area of the 90° C TL band of tobacco leaves plotted against time after treatment. Leaves were treated with a cryptogein solution (closed symbols) or with H_2O = control (open symbols). Same conditions as in Fig. 1.

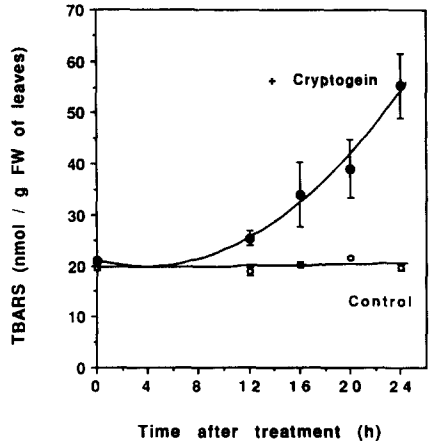

Fig. 4. Thiobarbituric acid reactive substances (TBARS) in tobacco leaves as expressed in nmol per g leaf (fresh weight) plotted against time after treatment. Leaves were treated with a cryptogein solution (closed symbols) or with H_2O = control (open symbols). Values are means ± S.E. averaged from two separate experiments with two repetitions each.

Low-temperature fluorescence spectra (Fig. 5) revealed an initial strong decline of the 684 nm PS II emission, followed by an approx. 10 nm down-shift of the 730 nm PS1 band and the emergence of a small peak at 705 nm which can be tentatively attributed to the emission of the disconnected PS I peripheral antenna [33]. At longer incubation times, a general broadening of the emission bands was observed, resulting in the disappearance of structured emission. In parallel, cryptogein-treated samples showed a marked reduction in the total chlorophyll content on a leaf dry weight basis, up to 60% at 24 h after treatment (data not shown).

PAM recordings of the fluorescence induction, with saturating light pulses, allowed us to calculate initial F_v/F_m and $\Delta F/F'_m$. In control leaves, F_v/F_m was approx. 0.84, for the whole time-period of the experiment, which corresponds to the value usually found in healthy leaves of

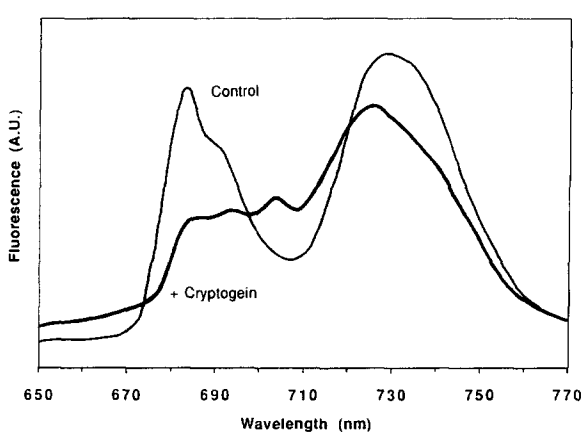

Fig. 5. Fluorescence emission spectra at 77 K of tobacco leaves recorded 20 h after treatment. Leaves were treated with a cryptogein solution (thick line) or with H_2O = control (thin line) and kept in the dark until measurement.

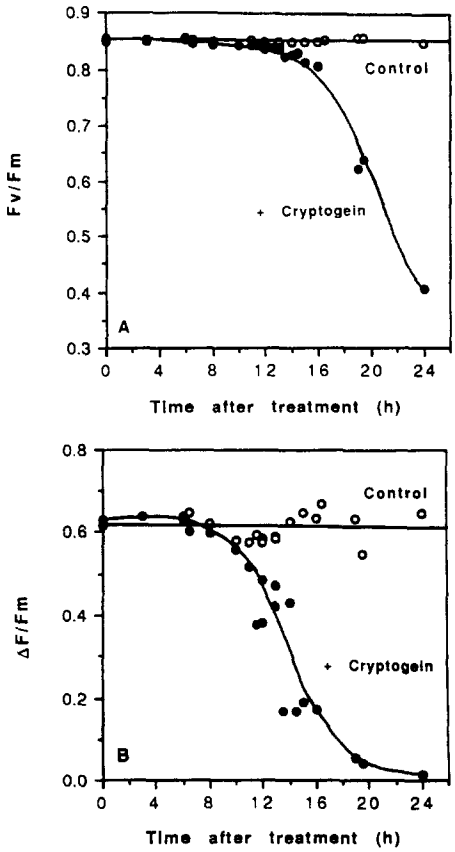

Fig. 6. F_v/F_m ratio (A) and $\Delta F/F_m$ ratio (B) of chlorophyll fluorescence of tobacco leaves plotted against time after cryptogein treatment. Leaves were treated with a cryptogein solution (closed symbols) or with H_2O = control (open symbols) and kept in the dark until measurement. Fluorescence was monitored by a pulse amplitude modulation (PAM) fluorometer as described in Materials and methods.

different plant species [34]. In treated leaves, F_v/F_m decreased progressively, down to 0.4 after 24 h (Fig. 6A). The photochemical quenching also fell, so that the $\Delta F/F_m$ ratio, an estimate of the quantum yield of PS II [35], was lowered from 0.61 to 0.2 after 16 h, exclusively in treated samples, under a 135 $\mu E\,m^{-2}\,s^{-1}$ continuous illumination (Fig. 6B). This indicates that the photosynthesis efficiency was strongly altered. It should be noted that a close negative correlation exists between both F_v/F_m and $\Delta F/F_m$ ratios with the band intensity of the 90° C TL band (c.c. = −0.926 and −0.874, resp.).

4. Discussion

Cryptogein treatment caused a considerable increase in the intensity of a high-temperature thermoluminescence band. This band did not originate from recombination of charges stored on the PS II electron carriers, since no difference was discernible when the leaf fragments were preilluminated or not. The T_m maximum was dependent on the heating-rate. This can be explained by the fact that the heat-induced formation of excited species related to lipid

peroxidation and transferring their energy towards chlorophyll is a time-dependent process. As a consequence, the pool of exciting molecules is depleted earlier during TL recording when heating is performed at a lower rate. The high-temperature band observed in tobacco leaves is similar to the 75° C band found in spinach and mung bean chloroplasts [20], which has been attributed by these authors to a temperature-induced interaction between molecular oxygen and the photosynthetic membrane in the course of a lipid peroxidation process. However, the present TBARS data indicate (at least in the case of leaves) that the occurrence of the chlorophyll-exciting compounds does not result from the heating of the samples but that it originates from preexisting differences between samples.

We were able to corroborate the relation between the high-temperature peak and overall lipid peroxidation by means of the TBARS data (Fig. 4). The TBA assay is the most widely used procedure to detect malondialdehyde (MDA) or MDA-like susbstances. Although it has often been criticized due to the interference by many other substances (which may explain the constant amount of TBARS found in control leaves), a comparison with other more direct methods was satisfactory enough to warrant its value in estimating lipid peroxidation [36].

The elevation of the level of constant fluorescence F_0 starting near 40° C can be explained in essence by a heat-induced perturbation of the thylakoid lipid bilayer [37]. Cryptogein treatments modify the shape of the F_0 peak (maximum at 52° C) and lower the critical temperature, T_c, indicating that the disturbance of the membrane is initiated at lower temperatures. This might originate from altered thylakoid lipid properties, tentatively due to the peroxidation of the (highly unsaturated) lipid hydrocarbon chains, and hence from a destabilization of the thylakoid membrane.

Fluorescence emission spectra confirm a progressive disorganization of the chlorophyll antenna, initially showing a decrease of the 684 nm PS II emission, followed by the progressive broadening and disappearance of other bands. The fact that the flash-induced TL emission (B band) is only slightly reduced during the same incubation period indicates that the cores of PS II and PS I are damaged more slowly than the antennae.

From the observed alterations of the lipid environment, it seemed inevitable that the functioning of the photosynthetic apparatus would also be affected.

Indeed, the area of the B band decreased progressively over the 24 h incubation time towards total disappearance. Furthermore, both the F_v/F_m ratio and the quantum efficiency measured by $\Delta F/F_m$ of PS II decreased in treated samples. Although leaves were maintained in the dark or in dim light, these reductions in the B band area [38] and in the F_v/F_m and $\Delta F/F_m$ ratios [39] are comparable with those induced by photoinhibitory treatments. This might be explained by the fact that both cryptogein and photoinhibition cause peroxidation reactions.

In conclusion, cryptogein treatment induces excited species within the chloroplast, most probably involved in the lipid peroxidation process, which can be detected through their heat-induced energy transfer to chlorophyll. The lipid breakdown process involves injury to the photosynthetic apparatus and PS II in particular. The possibility to study simultaneously, in intact leaf tissues, the accumulation of peroxidized species and their effect on the thylakoid membrane might provide useful information on the mechanisms of action of the hypersensitive response upon treatment with fungal elicitors. However, additional investigations are necessary to gain insight in the role of elicitor-induced peroxidative breakdown of the thylakoid membrane in relation to the damage induced in other cellular membranes.

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