Effect of Tobamovirus Infection on Thermoluminescence Characteristics of Chloroplasts from Infected Plants

Jaber Rahoutei[a], Matilde Barón[a,*], Isabel García-Luque[b], Magdolna Droppa[c], András Neményi[c] and Gábor Horváth[c]

[a] Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (C.S.I.C.), E-18008 Granada, Spain. Fax: +34-58-129600. E.mail: mbaron@eez.csic.es
[b] Department of Plant Biology, Centro Investigaciones Biológicas, C. S. I.C, E-28006 Madrid, Spain.
[c] Department of Plant Physiology, University of Horticulture and Food Industry, Budapest, P.O. Box 53, Hungary, H-1518.

* Author for correspondence and reprint requests

Z. Naturforsch. **54c,** 634–639 (1999); received November 15, 1998/January 15, 1999

Biotic Stress, Photosynthetic Electron Transport, Photosystem II, Thermoluminescence, Virus Infection

Changes of thermoluminescence characteristics as well as the O_2-evolving capacity was analysed in chloroplasts isolated from *Nicotiana benthamiana* infected with pepper and paprika mild mottle viruses and their chimeric hybrids. The electron transport activity in thylakoids of virus-infected plants was inhibited and could be restored by adding DPC or Ca^{2+} which indicated that the virus infection altered the oxygen-evolving complex. In thermoluminescence characteristics of plants infected with either viruses, the first well defined response was a shift in the peak position of the B band from 20 °C to 35 °C corresponding to $S_3(S_2)Q_B^-$ and $S_2Q_B^-$ charge recombinations, respectively, which showed an inhibition in the formation of higher S states in the water splitting system. Simultaneously, a new band appeared around 70 °C due to chemiluminescence of lipid peroxidation. Further progress of the viral infection dramatically decreased the intensity of bands originated from charge recombinations with a concomitant increase of the band at 70 °C indicating the general oxidative breakdown of injured thylakoids.

Introduction

Early studies about the effect of virus infection on photosynthesis summarized in a classical work of plant pathology (Goodman *et al.*, 1986) show that plant virus infection alter the photosynthetic process of their host plants. However, little progress on this research field has been achieved essentially due to the difficulties for controlling experimentally the consequences of biotic stress, which are highly variable (for a review, see Balachandran *et al.*, 1997 and references therein). In addition, an insight into plant pathology is always limited by the physiological understanding of the healthy host.

From the perspective of photosynthesis research, the host-virus interaction was examined using the contemporary knowledge of photosynthetic mechanisms (Naidu *et al.*, 1986; Hodgson and Beachy, 1989; Takahashi and Ehara, 1992). Advances in techniques for stress detection in the photosynthetic apparatus during the last decade have facilitated studies of early responses to infection given by host plants. Thus, Chl fluorescence measurements in different hosts infected with various viruses showed that photochemical efficiency of PSII was decreased by the viral infection (Balachandran *et al.*, 1994). The degree of PSII inhibition in virus-infected plants depended on both growing conditions of plants and viral strains. Viruses which did not induce symptoms in their host did not greatly affect PSII function. In contrast, viruses which were able to induce symptoms

Abbreviations: Chl, chlorophyll(s); DPC, 1,5-diphenylcarbazide; d.p.i., day post-inoculation; MV, methylviologen; OEC, oxygen-evolving complex; PaMMV, paprika mild mottle virus; PMMoV-S and -I, Spanish and Italian strains of pepper mild mottle virus; PSII, photosystem II; Q_A and Q_B, the primary and secondary electron acceptor of PSII; TL, thermoluminescence.

0939–5075/99/0900–0634 $ 06.00

in plants inhibited dramatically PSII functioning (van Kooten *et al.*, 1990).

It was proposed that the inhibition of photosynthetic electron transport by the tobamoviruses was caused by a direct interaction between specific viral gene products, such as the coat protein, and the PSII apparatus (Reinero and Beachy, 1989). In contrast, other authors proposed that the inhibition of the PSII activity has been associated to a reduced accumulation of PSII polypeptides of the OEC complex (Naidu *et al.*, 1994; Hodgson *et al.*, 1989; Takahashi and Ehara, 1991). In preliminary studies we have shown alterations of the photosynthetic process induced by several pepper strains of the tobamoviruses. Infected plants showed a decreased PSII activity associated to a reduction in the level of OEC proteins (Barón *et al.*, 1995).

However, the exact mechanism of the action of viral infection on PSII is still unknown. Although the sensitivity of PSII to viral infection at the level of electron transport is well documented, the mechanism of inhibition is still a matter of debate.

For a further characterization of the inhibitory mechanism of plant virus within PSII we have used in the present work thermoluminescence (TL), an emerging technique for stress studies on PSII. TL originates from the charge recombination between positively charged donors and negatively charged acceptors of PSII and even small changes in the redox properties of PSII electron transport are reflected in TL characteristics (see for review Horváth, 1986; Demeter and Govindjee, 1989; Vass and Govindjee, 1996; Inoue, 1996). TL has been proven a very useful method for analysing the damaging mechanisms of a number of abiotic environmental factors that affect the functioning of PSII such as photoinhibition by visible light and UV-B, heavy metals and extreme temperature (Ohad *et al.* 1988; Mohanty *et al.*, 1989; Desai, 1990; Allakhverdiev *et al.*, 1992; Hideg *et al.*, 1993; Vass and Govindjee, 1996; Vavilin *et al.*, 1998; Horváth *et al.*, 1998). Recently, TL emission bands appearing at higher temperatures (60–120 °C) were applied to study the host response in the hypersensitive reaction an incompatible pathogen-interaction (Stallaert *et al.*, 1995). These higher temperature TL components which do not originate from charge recombination in PSII, arise most likely from oxidative chemiluminescence of membrane lipids (Hideg *et al.*, 1993). Since their emission strongly increased in isolated thylakoids under stress treatment like extreme temperature, heavy metals and pathogen elicitors, the high temperature TL bands were proposed to be considered as stress indicators (Vavilin *et al.*, 1998). Since PSII is one of the target of biotic stress, we anticipated that TL could also be a tool for the detection and study of the effect of compatible host-pathogen interaction on photosynthetic electron transport.

Consequently in this work we have investigated the effect of the infection with tobamoviruses on PSII by analysing the thermoluminescence characteristics of functional thylakoids isolated from infected plants. In addition polarographic measurements of different Hill reactions were carried out to test the functionality of the OEC. As the host-virus system, we used *Nicotiana benthamiana* and different strains of pepper mild mottle virus (PMMoV) and paprika mild mottle virus (PaMMV), as well as, chimeric viruses with hybrid genomes of the former viruses.

Materials and Methods

Plant and viruses

Nicotiana benthamiana Gray plants were cultivated in a growth chamber at 200 μmol photons $\cdot m^{-2} \cdot s^{-1}$ with a 16 h photoperiod, a temperature regime of 25/20 °C (day/night) and a relative humidity of 80%. Plants at the developmental stage of 6–8 fully-expanded leaves were mechanically inoculated in the three lower leaves by dusting with carborundum and gently rubbing with 50 μl of virus-containing solutions per leaf (50 μg of virus/ml suspended in 20 mM sodium phosphate buffer pH 7.0), and rising with tap water. The origin of the viruses used, the Spanish (S) and Italian (I) strains of pepper mild mottle virus (PMMoV), paprika mild mottle virus (PaMMV) as well as their chimeric hybrids, THI-3 and THG-2, has been previously reported by Alonso *et al.* (1991) and Berzal *et al.* (1995). TL and electron transport measurements were carried out in thylakoid membranes isolated at different days post-inoculation (d.p.i.) from non-infected *Nicotiana benthamiana* as well as from plants infected with the different viruses.

Thylakoid isolation

Thylakoids were prepared as described by Arellano *et al.* (1995) and stored at high concentration (5–6 mg Chl/ml) at −80 °C in a buffer containing 50 mM 2-(N-morpholino ethanesulfonic acid MES, pH (6.5) 15 mM NaCl, 5 mM $MgCl_2$ and 400 mM sucrose.

Electron transport measurements

Photosynthetic electron transport was measured polarografically in a Clark type O_2 electrode. To test the functionality of the electron transport chain we estimated the extent of the oxygen uptake associated to the Hill reactions $H_2O \rightarrow MV$ and $DPC \rightarrow MV$. The assay medium for $H_2O \rightarrow MV$ reaction contained 20 mM MES (pH 6.5), 300 mM sucrose, 10 mM NaCl, 0.05% bovine serum albumin, 0.15 mM MV as electron acceptor and thylakoids equivalent to 17 μg Chl/ml. Measuring $DPC \rightarrow MV$ reaction, the assay medium was supplemented with 0.5 mM DPC. In addition, for investigating if the decreased electron transport due to the depletion of OEC proteins could be restored by Ca^{2+}, the thylakoid membrane pellet was suspended in the assay medium containing 5 mM $CaCl_2$, incubated at 4 °C for 20 min, and the $H_2O \rightarrow MV$ electron transport activity was measured.

TL measurements

Before TL measurements, samples were diluted with the buffer used for the storage to the Chl equivalent of 250 μg/ml. 0.5 ml thylakoid suspension (pH 6.5) containing 125 μg Chl was measured using an apparatus similar to that described earlier (Demeter *et al.*, 1979). The light source was a 650 W tungsten lamp provided 10 W/m² intensity at the surface of the sample. Samples were illuminated during cooling from +20 °C to −60 °C. TL was measured during heating the sample in darkness with a constant heating rate of 20 °C/min.

Results and Discussion

We tested the thermoluminescence properties of thylakoid membranes isolated from control *Nicotiana benthamiana* plants and those infected with different tobamoviruses at 7, 14 and 21 d.p.i. In thylakoids isolated from healthy plants, both the A and B thermoluminescence bands appeared (Fig. 1A). As shown previously, the A band which appears between −30 and −10 °C is due to $S_3Q_A^-$ charge recombination, while the B band at around +20 °C is an integration of two overlapping bands, namely B_1 (+17 °C) and B_2 (+37 °C) bands corresponding to $S_3Q_B^-$ and $S_2Q_B^-$ recombination, respectively (for review see Horváth *et al.*, 1986; Demeter and Govindjee, 1989).

In thylakoids isolated from plants infected with either PMMoV-S or PMMoV-I, neither the A nor the B bands showed any specific responses to the viral infection up to 7 d.p.i. (Fig. 1B). At 14 d.p.i., the intensity of the A band did not change significantly with either virus (Fig. 1C). In contrast, the B band,which represented $S_3(S_2)Q_B^-$ showed an upward shift in its peak position indicating changes in the redox span between the positive and negative charges located on redox partners generating this band. As it was shown previously, a similar but downward shift in the peak position of the B band indicated a transition from $S_2Q_B^-$ to $S_3Q_B^-$ charge recombination (Horváth *et al.*, 1998). Simultaneously with the shift in the peak position of the B band, a new band at 70 °C appeared (Fig. 1C) which probably corresponded to the TL band at 75 °C described by Hideg and Vass (1993). This band was attributed to a temperature-induced interaction between molecular oxygen and the photosynthetic membrane in the course of

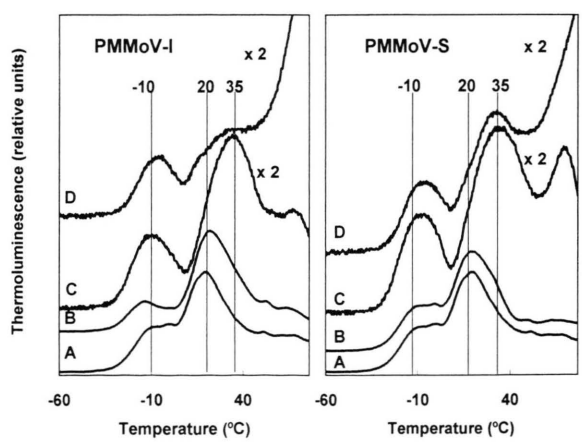

Fig. 1. Alterations in TL characteristics of thylakoids isolated at different days post-inoculation (d.p.i.) from *Nicotiana benthamiana* infected with two different strains of pepper mild mottle virus. A: control; B: 7 d.p.i.; C: 14 d.p.i.; D: 21 d.p.i. The C and D curves were magnified by the factor of 2.

lipid peroxidation processes during heating of the sample. At 21 d.p.i., the intensity of both the A and B bands was dramatically reduced and the intensity of the 70 °C band was increased enormously, associated with a shift toward higher temperatures (Fig1. D). Very similar tendencies were found with either PaMMV virus strain (data not shown) or with both chimeric hybrids at any d.p.i. investigated (Fig. 2).

The fact that the intensity of the bands which originates from charge recombination was decreased by the progress of viral infection indicated the inhibition of PSII function. TL studies by Vass *et al.* (1992) reflected that the ability of OEC complex to reach its higher oxidation states was limited when some OEC proteins were absent. Although these proteins are not an absolute requirement for water oxidation, their absence disturbs the redox cycling of the OEC complex and retards the formation of higher S states. As we demonstrated in a previous work, the viral infection resulted in disturbances on the OEC proteins (Barón *et al.*, 1995). In thylakoids isolated from plants infected with viruses used in the present study, the levels of both the 24 and 16 kDa extrinsic proteins of the OEC were reduced to various degrees. Consequently, PSII-mediated electron transport decreased, and the loss of the OEC extrinsic proteins affected the oxygen evolution rate of thylakoid membranes isolated from infected plants. As Homann and Madabusi (1993) demonstrated in Ca-depleted PSII membranes devoided of the 17 and 23 kDa polypeptides, the B band was emitted at a higher temperature due to the stabilization of an abnormally stable S_2 state. The shift in the peak position of the B band observed at 14 d.p.i. and thereafter could also be the result of changes within OEC.

We assumed that if the loss of the 24 and 16 kDa polypeptides of the OEC induced by the viral infection was responsible for the change observed in TL characteristics, it had to be associated to a decrease of the oxygen-evolving capacity. Therefore, in order to confirm this assumption we measured the functionality of the electron transport chain (Fig. 3). In addition, we carried out assays in the presence of DPC, an artificial electron donor that could bypass the OEC, as well as, we investigated whether the decreased electron transport activity due to the depletion on the OEC proteins could be restored by Ca addition.

When the activity of the whole electron transport chain was measured by the Hill reaction $H_2O \rightarrow MV$ in thylakoids isolated from infected plants at 21 d.p.i., the inhibition was between 25 and 70%, depending on the virus strain. In all virus infected samples, the PSII electron transport activity was partially restored either by DPC or Ca^{2+}, confirming that the damage might be, in part, affecting the OEC. Similar results were obtained by Reinero and Beachy (1989) and Taka-

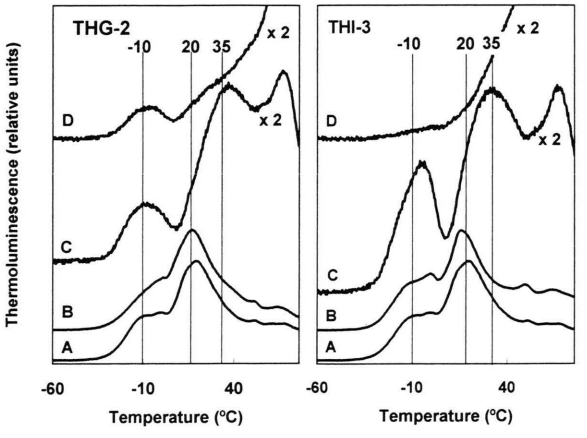

Fig. 2. Changes in TL characteristics of thylakoids isolated at different days post-inoculation (d.p.i.) from *Nicotiana benthamiana* infected with the two chimeric hybrids between pepper and paprika mild mottle viruses. A: control; B: 7 d.p.i.; C: 14 d.p.i.; D: 21 d.p.i. The C and D curves were magnified by the factor of 2.

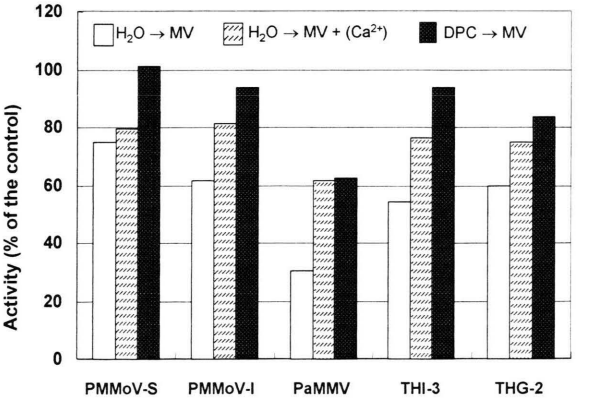

Fig. 3. The electron transport activity as well as its possible restoration with DPC or Ca^{2+} addition in thylakoids infected with different virus strains at the 21 day post-inoculation.

hashi and Ehara (1992) in TMV- and CMV-infected plants, respectively.

In our experiments, another demonstrative change in the TL characteristics of thylakoids in the latter stages of the infection was manifested by the appearance and increase of the TL band over 70 °C. Besides the TL components which originate from charge recombination in PSII, there were other components which appeared at high temperature and most likely arose from oxidative damage of membrane lipids (Hideg and Vass, 1993; Stallaert *et al.*, 1995; Vavillin *et al.*, 1998) indicating a general disorganization of thylakoid membranes in the last stage of the virus disease. As Stallaert *et al.* (1995) demonstrated, the amplitude of this band was stepwise increased by the progress of the infection in the host. Similarly in our experiments, a dramatic increase in the intensity of the band at 70 °C was observed between 14 and 21 d.p.i.

Since the symptomatic and asymptomatic leaves from the same plant at the same d.p.i. represent two different developmental stages of viral infection, the above described differences in TL characteristics have also to be reflected in the two types of leaves. As shown in Fig. 4, the glow curves of both the parental virus (PaMMV) and the chimeric hybrid (THG-2) exhibited, indeed, the expected response in a very similar way. At 14 d.p.i, the asymptomatic leaves already exhibited the characteristic shift of the B band to 35 °C and had only a shoulder at 70 °C. In the symptomatic leaves, however, which represent a later stage of viral infection, the B band was reduced and the high temperature band become dominant. The increase in this high temperature band has always been associated to the appearance of visible symptoms. This is in accordance with the earlier finding of Stallaert *et al.* (1995) who detected the dominance of the high temperature band in leaves displaying hypersensitive reaction. They concluded that the appearance of the high temperature band represented the peroxidative breakdown of thylakoids and it can be considered as stress indicator. The fact that in our experiments the shift of the peak position of the B band always preceded the appearance of the high temperature band suggested that in leaves having no visible symptoms,

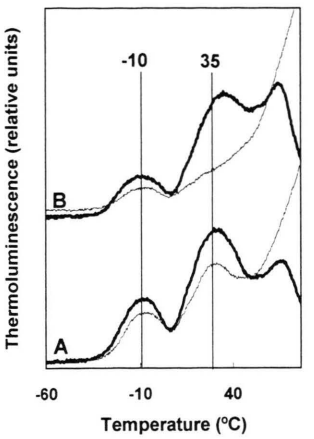

Fig. 4. TL characteristics of thylakoids isolated from asymptomatic (black lines) and symptomatic (grey lines) leaves from *Nicotiana benthamiana* plants infected with either the paprika mild mottle virus PaMMV (A) or the chimeric hybrid THG-2 (B) at 21 day post-inoculation (d.p.i).

the $B_1 \rightarrow B_2$ peak transition can be used as a stress indicator prior to the appearance of the high temperature band associated with visible symptoms.

In the present paper we have demonstrated that viral infection strongly influenced the TL characteristics of thylakoids by affecting the donor side of PSII. On the one hand, viral infection shifted the $S_3(S_2)Q_B^-$ to $S_2Q_B^-$ charge recombination responsible for the B band generation prior to the appearance of the usual visible symptoms. At this stage of the infection, the OEC is damaged and resulted in a decrease of the O_2-evolving capacity which can partially be restored by the addition of DPC or Ca^{2+}. On the other hand, further progress of the infection associates with the appearance of a new temperature band around 70 °C which represents a general oxidative breakdown of injured thylakoids.

Acknowledgements

This research was supported by the Hungarian-Spanish Intergovermental Exchange Programme (E-19/97), the Hungarian National Scientific Research Found (OTKA T026075, T026078), the Foundation for the Hungarian Higher Education and Culture (166/98) and the Spanish DGICYT (PB94-0116, BIO98-0860-CO2-02 and BIO98-0860-CO2-01).

Allakhverdiev S. I., Klimov V. V. and Demeter S. (1992), Thermoluminescence evidence for light-induced oxidation of tyrosine and histidine residues in manganese-depleted photosystem II particles. FEBS Lett. **297**, 51–54.

Alonso E., García Luque I., de la Cruz A., Wicke B., Ávila Rincón M. J., Serra M. T., Castresana C. and Díaz-Ruíz J. R. (1991), Nucleotide sequence of the genomic RNA of pepper mild mottle virus, a resistance-breaking tobamovirus in pepper. J. Gen. Virol. **72**, 2875–2884.

Arellano J. B., Schröder W., Chueca A. and Barón M. (1994), Removal of nuclear contaminants and of non-specifically bound PSII copper from PSII preparations. Physiol. Plant. **91**, 369–374.

Balachandran S., Osmond C. B. and Daley F. P. (1994), Diagnosis of the earliest strain-specific interactions between tobacco mosaic virus and chloroplasts of tobacco leaves *in vivo* by means of chlorophyll fluorescence imaging. Plant Physiol. **104**, 1059–1065.

Balachandran S., Osmond C. B. and Daley F. P. (1997), Concepts of plant biotic stress. Some insights into the stress physiology of virus-infected plants, from the perspective of photosynthesis. Physiol. Plant. **100**, 203–213.

Barón M., Rahoutei J., Lázaro J. J. and García Luque I. (1995), Photosystem II response to biotic and abiotic stress. In: Photosynthesis from Light to Biosphere (P. Mathis, ed.), Vol. **4**. Kluwer Academic Publishers, The Hague, The Netherlands, pp. 897–901.

Berzal-Herranz A., De la Cruz A., Tenllado F., Diaz-Ruiz J. R., López L., Sanz A. I., Vaquero C., Serra M. T. and García-Luque I. (1995), The *Capsicum* L3 gene-mediated resistance against the tobamoviruses is elicited by the coat protein. Virology **209**, 498–505.

De la Cruz A., López L., Tenllado F., Díaz-Ruíz J. R., Sanz A. I., Vaquero C., Serra M. T. and García-Luque I (1997), The coat protein is required for the elicitation of the *Capsicum* L2 gene-mediated resistance against the tobamoviruses. Mol. Plant-Microbe Interact. **10**, 107–113.

Demeter S., Herczeg T., Droppa M. and Horváth G. (1979), Thermoluminescence characteristics of granal and agranal chloroplasts of maize. FEBS Lett. **100**, 321–324.

Demeter S. and Govindjee (1989), Thermoluminescence in plants. Physiol. Plant. **75**, 121–130.

Desai T. S. (1990), Studies on thermoluminescence, delayed light emission and oxygen evolution from photosynthetic materials: UV effects. Photosynth. Res. **25**, 17–24.

Goodman R. N., Kiraly Z. and Wood K. R. (1986), Photosynthesis. In: The Biochemistry and Physiology of Plant Disease. University of Missouri Press, Columbia, pp. 46–74.

Hodgson A. R., Beachy N. R. and Pakrasi B. H. (1989), Selective inhibition of photosystem II spinach by tobacco mosaic virus: an effect of the viral coat protein. FEBS Lett. **245**, 267–270.

Homan P. H. and Madabusi L. V. (1993), Modification of the thermoluminescence properties of Ca^{2+}-depleted photosytem II membranes by the 23 kDa polypeptide and by oligocarboxylic acids. Photosynth. Res. **35**, 29–39.

Horváth G. (1986), Usefulness of thermoluminescence in herbicide research. Crit. Rev. Plant Sci. **4**, 293–310.

Horváth G., Arellano J. B., Droppa M. and Barón M. (1998), Alterations in photosystem II electron transport as revealed by thermoluminescence of Cu-poisoned chloroplasts. Photosynth. Res. **57**, 175–181.

Hideg É., Sass L., Barbato R. and Vass I. (1993), Inactivation of oxygen evolution by UV-B irradiation. A thermoluminescence study. Photosynth. Res. **38**, 455–462.

Inoue I. (1996), Photosynthetic thermoluminescence as a simple probe of Photosystem II electron transport I. In: Biophysical Techniques on Photosynthesis (J. Amesz and A. J. Hoff, eds.). Kluwer Academic Publishers, The Netherlands, pp. 93–107.

Mohanty N., Vass I. and Demeter S. (1989), Copper toxicity affects PSII electron transport at the secondary quinone acceptor (Q_B). Plant Physiol. **90**, 175–179.

Naidu R. A., Krishnan M., Nayudu M. V. and Gnanam A. (1986), Studies on peanut green mosaic virus infected peanut (*Arachis hypogaea* L.) leaves. III. Changes in the polypeptides of photosystem II particles. Physiol. Mol. Plant Pathol. **29**, 53–58.

Ohad I., Koike H., Shochat S. and Inoue Y. (1988), Changes in the properties of reaction center II during the initial stages of photoinhibition as revealed by thermoluminescence measurements. Biochim. Biophys. Acta **933**, 288–298.

Reinero A. and Beachy N. R. (1989), Reduced photosystem II activity and accumulation of viral coat protein in chloroplasts of leaves infected with tobacco mosaic virus. Plant Physiol. **89**, 111–116.

Stallaert V. M., Ducruet J.-M., Tavernier E. and Blein J. P. (1995), Lipid peroxidation in tobacco leaves treated with the elicitor cryptogein: evaluation by high-temperature thermoluminescence emission and chlorophyll fluorescence. Biochim. Biophys. Acta **1229**, 290–295.

Takahashi H. and Ehara Y. (1988), Changes in the activity and the polypeptide composition of the oxygen-evolving complex in photosystem II of tobacco leaves infected with cucumber mosaic virus strain Y. Mol. Plant-Microbe Interact. **1**, 243–249.

Takahashi H., Ehara Y. and Hirano H. (1991), A protein in the oxygen-evolving complex in the chloroplast is associated with symptom expression on tobacco leaves infected with cucumber mosaic virus strain Y. Plant Mol. Biol. **16**, 689–698.

van Kooten O., Meurs C. and van Loon L. C. (1990), Photosynthetic electron transport in tobacco leaves infected with tobaco mosaic virus. Physiol. Plant. **80**, 446–452.

Vass I. Cook K. M., Deák Z., Mayes S. R. and Barber J. (1992), Thermoluminescence and flash-oxygen characterization of the IC2 deletion mutant of *Synechocystis* sp. PCC 6803 lacking the photosystem II 33 kDa protein. Biochim. Biophys. Acta **1102**, 195–201.

Vass I. And Govindjee (1996), Thermoluminescence from the photosynthetic apparatus. Photosynth. Res. **48**, 117–126.

Vavilin D. V., Ducruet J. M., Matorin D. N., Venediktov P. S. and Rubin A. B. (1998), Membrane lipid peroxidation, cell viability and photosystem II activity in the green alga *Chlorella pyrenoidosa* subjected to various stress conditions. J. Photochem. Photobiol. **42**, 233–239.