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Photosynthesis in leaves of *Nicotiana tabacum* L. infected with tobacco mosaic virus

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Abstract

In tobacco leaves inoculated with tobacco mosaic virus (TMV), changes in chlorophyll (Chl) and carotenoid contents, parameters of slow Chl fluorescence kinetics, *i.e.* the maximum quantum yield of photosystem (PS2) photochemistry F_v/F_m, the effective quantum yield of photochemical energy conversion in PS2 Φ_2, ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2 F_v/F_0, non-photochemical quenching (NPQ), and photochemical activities of isolated chloroplasts from systemically infected tobacco leaves were investigated. We compared two successive stages of infection, the first in the stage of vein clearing at 9th day post inoculation (dpi) and the second at 22nd dpi when two different regions, *i.e.* light- (LGI) or dark-green (DGI) islands in the infected leaf were apparent and symptoms were fully developed. These two different regions were measured separately. The Chl and carotenoid contents in infected leaves decreased with a progression of infection and were lowest in LGI in the second stage. Also the ratio of Chl *a/b* declined in similar manner. The maximum quantum yield of PS2 photochemistry F_v/F_m, was decreased in the following order: first stage, DGI, and LGI. The same is true for the ratio F_v/F_0. The decrease of Φ_2 in infected leaves declined as compared to their controls. On the contrary, NPQ increased in infected leaves, the highest value was found in the first infection stage. Photochemical activities of the whole electron transport chain in isolated chloroplasts dramatically declined with the progression of symptoms, the lowest value was in LGI. Similarly, but to a lesser extent, the activity of PS2 in isolated chloroplasts decreased in infected leaves. Generally, the most marked impairment of the photosynthetic apparatus was manifested in the LGI of infected leaves.

Additional key words: carotenoids; chlorophyll; chlorophyll fluorescence; electron transport; photosynthetic efficiency; photosystem 2; tobacco.

Introduction

Viruses, such as TMV, cause severe biotic stress to infected leaves leading to oxidative stress. Visual symptoms of viral infection are caused by chlorosis of leaf tissues. Metabolism of infected leaf tissue is strongly affected leading to the damage to the whole plant (*e.g.* Goodman *et al.* 1967, Šindelářová *et al.* 1997, 2000, Šindelář and Šindelářová 2002). As some TMVs multiply in a plant the disease becomes visible as numerous light and dark green islands within the leaf tissue creating a clear-cut mosaic (Fulton 1951, Atkinson and Matthews 1967, Hanušová *et al.* 1990, Guo and Garcia 1997, Yelina *et al.* 2002). The virus content is higher in light spots and it interferes there with chloroplast development and function (Goodman *et al.* 1986). Marked reduction in photosynthetic activity arises early after inoculation in association with reduced size and number of chloroplasts, reduced chlorophyll (Chl) content and low efficiency of CO_2 fixation in the chloroplasts (Jensen 1968, Tu *et al.* 1968). The severity of damage to a leaf depends principally on a strain or a mutant of a virus. The impairment of

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Abbreviations: Chl – chlorophyll; DGI – dark green islands; dpi – days post inoculation; ET – electron transport chain; F_m – maximum Chl fluorescence from dark-adapted leaves; F_m' – maximum Chl fluorescence from light-adapted leaves; F_0 – minimum Chl fluorescence from dark-adapted leaves; F_s – steady state Chl fluorescence from light-adapted leaves; F_v – variable Chl fluorescence from dark-adapted leaves; F_v/F_0 – maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2; F_v/F_m – maximum quantum yield of PS2 photochemistry; FeCy – potassium ferricyanide; LGI – light green islands; NPQ – non-photochemical quenching; PAM – pulse amplitude modulation; PS2 – photosystem 2; RC – reaction centre; TMV – tobacco mosaic virus; Φ_2 – effective quantum yield of photochemical energy conversion in PS2.
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chloroplast function is often coupled with a presence of viral coat protein in these organelles. This protein was found both in stroma and thylakoids of chloroplasts in systematically infected leaves (Reinero and Beachy 1986). Protein analysis indicated that in TMV infected spinach coat protein was associated with thylakoids, particularly with the photosystem 2 (PS2) fraction (Hodgson *et al*. 1989). Virus strain TMV-PV42 causing mild symptoms induced ten times less accumulation of coat protein in chloroplasts as compared to those from leaves infected with invasive strain of TMV PV230 (Reinero and Beachy 1989). Moreover, the activity of PS2 was inhibited in leaves infected with an aggressive strain while no reduction was found in PS2 activity in plants infected with a milder strain. Surprisingly, both viruses were found to replicate equivalently and their contents in a leaf were about the same.

Montalbini and Lupattelli (1986) compared two tobacco cultivars differing in susceptibility to TMV infection. The rate of whole electron transport in isolated chloroplasts from susceptible tobacco was not influenced by the infection whereas in chloroplasts from hypersensitive plants this rate was strongly inhibited. Van Kooten *et al*. (1990) investigated the change in electron transport in relation to symptom expression and deduced that quantum yields of PS2 electron transport rate were significantly diminished in virus strains inducing loss of Chl. Further, they found that PS2 was irreversibly damaged in the inoculated leaves and the ones directly above them reflecting an increased susceptibility to photoinhibition. Interestingly, both maximum photochemical efficiency (F_v/F_m) in isolated chloroplasts from TMV infected spinach as well as photochemical activity of PS2 were reduced as compared to controls but activity of PS1 was not affected by the infection (Hodgson *et al*. 1989).

In order to establish the relation between photosynthetic activity and virus content we studied the effects of TMV on photosynthetic capacity during progression of viral infection in tobacco leaves, namely photochemical efficiency of PS2 and the photochemical activities of the whole electron transport chain and PS2.

Materials and methods

Plant cultivation and virus inoculation: Two-month-old tobacco plants (*Nicotiana tabacum* L. cv. Samsun) grown under constant conditions in soil at an irradiance of 60 μmol(photon) m^{-2} s^{-1}, 16-h photoperiod, and average temperature of 25 °C, were used. Two leaves of the bottom insertion (approximately 5 cm long) were mechanically inoculated with purified tobacco mosaic virus (TMV, common strain) (Gooding and Hebert 1967) at a concentration of 100 g m^{-3}. Corresponding leaves of control plants were mock-inoculated with distilled water. The samples of systemically infected leaves and corresponding leaves from mock-inoculated plants were collected in the first stage on the 9th day post inoculation (dpi) when the first symptoms of infection appeared (vein clearing). Samples were further collected in the second stage on the 22nd dpi when two regions ("islands") differing in their tone of green colour were distinguished. The apparent light-green (LGI) and dark-green (DGI) regions were cut out with a scalpel and everywhere estimated separately (except Chl fluorescence parameters). The day of inoculation was designated as day zero.

Determination of TMV content: TMV content was determined by the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-TMV antibodies and alkaline phosphatase labelled antibodies raised against our isolate of TMV (common strain).

Contents of Chls and carotenoids were estimated by extracting 1 cm^2 of the leaf material in 3 cm^3 of dimethylformamide. The samples were incubated for 48 h in the dark at room temperature and then the absorbance of the extract was recorded at wavelengths of 480.0, 646.8, and 663.8 nm (*Hitachi U 3300*, Tokyo, Japan). The contents of Chls and carotenoids were calculated by the method of Porra *et al.* (1989).

Chl fluorescence parameters: Photochemical efficiency of electron transport through PS2 was specified from Chl fluorescence induction kinetics. It was measured after a 15 min dark period with the *PAM* chlorophyll fluorometer (*Walz*, Effeltrich, Germany) on fresh whole leaves at room temperature (Procházková and Wilhelmová 2004). In the second stage of infection, the areas of DGI or LGI were measured separately on the whole leaf. Measuring irradiance was 0.35 μmol m^{-2} s^{-1}, actinic irradiance 200 μmol m^{-2} s^{-1}, 700 ms saturated flash of 2 500 μmol m^{-2} s^{-1}. The *DA 100* data acquisition system (*Walz*, Effeltrich, Germany) was used for sampling and calculation. The Chl fluorescence ratio F_v/F_m ($F_v = F_m - F_0$) was calculated using nomenclature of van Kooten and Snel (1990). The ratio F_v/F_0, non-photochemical quenching $NPQ = (F_m - F_m')/F_m'$, effective quantum yield of photochemical energy conversion in PS2 $\Phi_2 = \Delta F/F_m'$ were determined according to Roháček (2002).

Chloroplasts were isolated according to Pospíšilová *et al*. (1998). Leaf blades (or tissue of LGI or DGI) were sliced and blended in isolation medium [mM]: HEPES 50 (pH 7.8), sorbitol 350, $MnCl_2$ 1, $MgCl_2$ 1, EDTA 2, K_2HPO_4 0.5. After filtration and following centrifugation (2 000×*g*, 10 min), the pellet was washed in re-suspending medium [mM]: *Tricine 50* (pH 7.5), sorbitol 330, $MnCl_2$ 1, $MgCl_2$ 1, EDTA 2. After repeated centrifugation the chloroplasts were re-suspended in a small volume of re-suspending medium to a Chl concentration

of *ca*. 1 kg m^{-3}.

Photochemical activities: Activities of the whole electron transport chain and of PS2 were measured as oxygen evolution using an oxygen electrode (*Hansatech*, King´s Lynn, UK). The suspension of chloroplasts was diluted in the assay medium [mM]: *Tricine 40* (pH 7.5), NaCl 2, $MgCl_2$ 5, K_2HPO_4 2. Final Chl concentration in the measuring chamber was 50 g m^{-3}. Potassium ferricyanide (FeCy) at a final concentration of 6.25 mM was present for estimation of the activity of the whole electron transport chain. For PS2 activity measurement, 1,4-phenylene diamine (PD) was added to a final concentration of 0.5 mM. The measurements were carried out at 25 °C and an irradiance of 1 000 μmol m^{-2} s^{-1} (for details see Pospíšilová *et al*. 1998).

Statistical treatment and chemicals: The results in the figures are presented as arithmetical means (±standard error of mean) of 3–7 measurements in two independent experiments with four measurement repetitions. The *t*-test was employed to characterise the differences between control and infected leaves as well as between DGI and LGI. All biochemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

Results

We investigated the concentrations of Chls and carotenoids and several photochemical parameters in tobacco leaves at two different stages of viral infection. The first one, 9 dpi, was characterised by vein clearing, the second stage, 22 dpi, was characterised by an occurrence of dark and light regions on leaves. The first leaf above the inoculated one was used for all measurements. Total Chl content at the stage of vein clearing was decreased by 12.7 % in infected plants compared to that of healthy controls (Fig. 1*A*). Also Chl *a/b* decreased by 3.4 % (Fig. 1*C*). Carotenoid content was lowered by 7.6 % of controls (Fig. 1*E*). Chl/carotenoid ratio decreased by 7.4 % (Fig. 1*G*). Maximum photochemical efficiency expressed as F_v/F_m decreased by 3.0 % in infected leaves (Fig. 2*A*). Ratio of variable to minimum Chl fluorescence yield (F_v/F_0) decreased by 5.0 % (Fig. 2*C*). Effective quantum yield of photochemical energy conversion in PS2 (Φ_2) decreased by 6.2 % (Fig. 2*E*). On the other hand, non-photochemical chlorophyll quenching (NPQ) increased by 46.4 % in infected leaves (Fig. 2*G*). The whole chain electron transport rate in isolated chloroplasts was diminished by 56.6 % as compared to controls already in first stage of infection. The photochemical activity of PS2 in isolated chloroplasts was reduced to even higher extent (by 60.3 %) compared to control in this stage of infection (Fig. 3*A*).

The differences between control and infected plants were much more striking at the second stage of viral infection, when two kinds of leaf regions differing in green colour – dark green islands (DGI) and light green islands (LGI) developed. They were assayed separately. Total Chl content decreased by 19.4 % in DGIs and by 45.0 % in LGIs of healthy control plants (Fig. 1*B*). Chl *a/b* decreased by 12.5 % in DGIs and by 15.4 % in LGIs (Fig. 1*D*). Carotenoid content decreased by 16.1 % and by 40.8 % in DGIs and LGIs, respectively (Fig. 1*F*). Chl/carotenoid ratio was diminished by 3.2 % in DGIs and by 6.6 % in LGIs, respectively (Fig. 1*H*). F_v/F_m declined by 5.6 % in DGIs and by 8.3 in LGIs (Fig. 2*B*) compared to controls. F_v/F_0 was reduced by 20.5 % in DGIs and by 31.6 % in LGIs (Fig. 2*D*). Also Φ_2 decreased by 4.4 % in DGIs and by 16.7 % in LGIs (Fig. 2*F*). On the contrary, NPQ increased by 9.9 % in

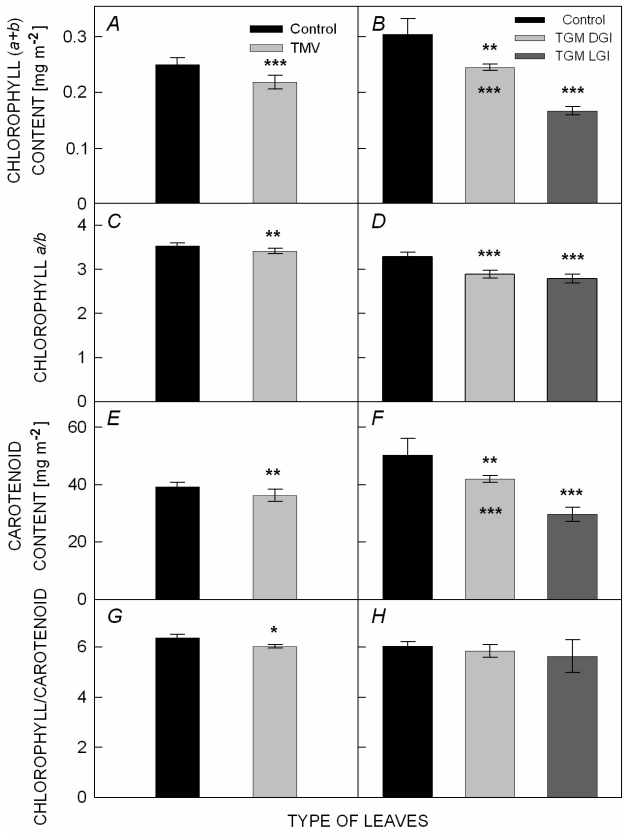

Fig. 1. Photosynthetic pigments in healthy control and infected tobacco leaves: chlorophyll (Chl) *a+b* content (*A,B*), chlorophyll *a/b* ratio (*C,D*), carotenoid content (*E,F*), and total Chl/carotenoid ratio (*G,H*) in the stage of vein clearing (*A, C, E, G*) and the second stage of dark-green (DGI) or light-green (LGI) islands (*B, D, F, H*). Means ± SE (*vertical bars*). *Asterisks above the bars* indicate significance of differences from the controls according to Student's *t*-test: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. *Asterisks inside a light grey column* represent significance between DGI and LGI in the same manner as the former ones.

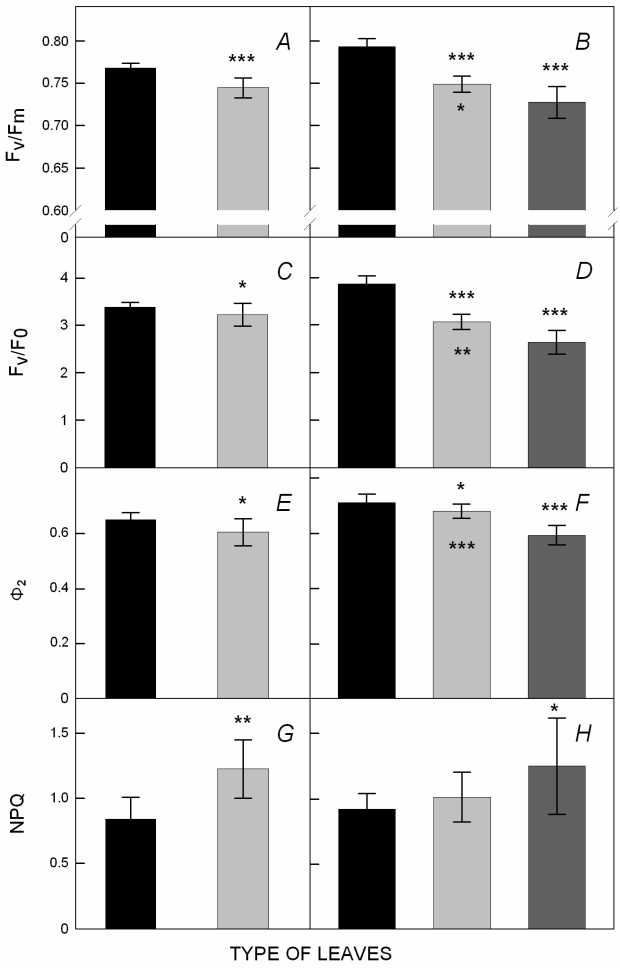

Fig. 2. Maximum photochemical efficiency, *i.e.* F_v/F_m (*A, B*), maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2, *i.e.* F_v/F_0 (*C, D*), effective quantum yield of photochemical energy conversion in PS2, *i.e.* Φ_2 (*E, F*), and non-photochemical quenching NPQ (*G, H*) in healthy control and infected tobacco leaves in the first stage of vein clearing (*A, C, E, G*), and in the second stage of dark- (DGI) and light-green (LGI) islands (*B, D, F, H*), respectively. The tissues of differently green islands were determined separately (DGI or LGI). Means ± SE (*vertical bars*). *Asterisks above the bars* indicate significance of differences from the controls according to Student's *t*-test: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. *Asterisks inside a light grey column* represent significance between DGI and LGI in the same manner as the former ones.

DGIs and by 35.9 % in LGIs of controls (Fig. 2*H*). In the second stage when regions visually differing by their Chl content were apparent, the reduction of photochemical activities in isolated chloroplasts was even more pronounced than in the first stage. In DGI the decline was

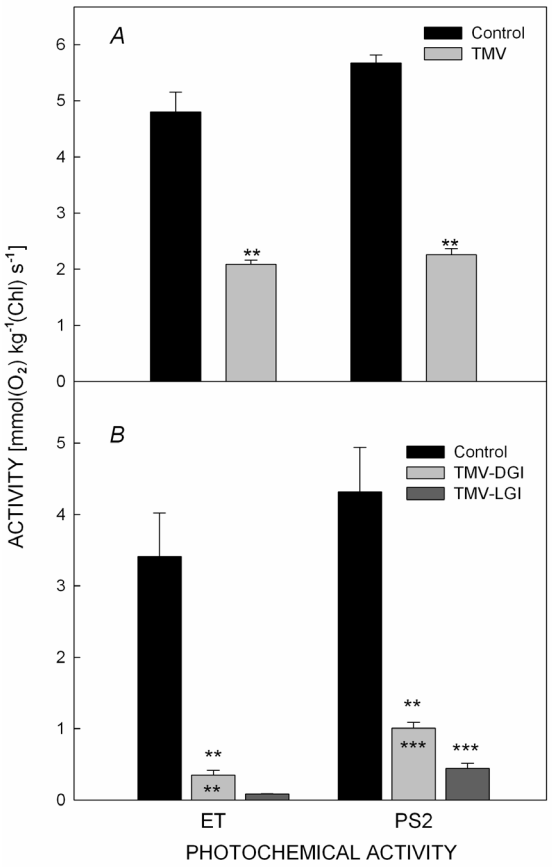

Fig. 3. Photochemical activities of isolated chloroplasts of the whole electron transport chain (ET) with potassium ferricyanide and of PS2 with 1,4-phenylene diamine as electron acceptors in control and infected tobacco leaves in the stage of vein clearing (*A*) and in infected tobacco leaves in the stage of dark-light green islands (*B*). The determinations in the second stage were done separately in dark-green islands (DGI) or light-green islands. *Asterisks above the bars* indicate significance of differences from the controls according to Student's *t*-test: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. *Asterisks inside a light grey column* represent significance between DGI and LGI in the same manner as the former ones.

by 89.7 and 76.7 % of controls for the whole electron transport rate and PS2 activity, respectively. In LGI we observed decrease by 98.1 and 90.0 % of control value for the whole electron transport rate and PS2 activity, respectively (Fig. 3*B*).

Content of TMV, estimated on the base of ELISA, increased starting from stage of vein clearing from 0.41 to 0.87 (2^{nd} DGI) and 2.02 (2^{nd} LGI). Hence it was 2.3 times higher in LGI than DGI which is in accordance with previous findings (Atkinson and Matthews 1970, Hanušová *et al.* 1990, Šindelářová and Šindelář 2004).

Discussion

The effects of virus infection on photosynthesis were studied by several authors in various host plants (Buchanan *et al*. 1981, Hodgson *et al.* 1989, Reinero and Beachy 1989, Rahoutei *et al*. 2000, Ryšlavá *et al*. 2003). Plant viruses reduced photosynthesis and increased susceptibility to photoinhibition. The viral coat protein is a frequent candidate for the interaction with components of PS2 and it may be responsible for impairing the function of photosynthesis (Hodgson *et al*. 1989, Banerjee *et al*. 1995).

We found that the total Chl content declined already at the first stage, then progressed further and was in LGI only about half as compared to controls. This is in agreement with observations that the virus is largely confined to the light areas, where it seems to interfere with chloroplast development and function (Goodman *et al*. 1986). We assume from the concomitant decrease of the Chl *a/b* ratio with the infection progress that the Chl *a* was preferentially degraded, namely in LGI. These data indicate that the Chl *a* in RCs is broken down faster than the light-harvesting pigment antennas that possess a Chl *a*/*b* ratio of 1.1–1.3 (Lichtenthaler *et al*. 1982). Interestingly, a similar trend was found in the carotenoid content, but the decrease was lower. The reduction of protection against active oxygen species in thylakoid membranes can be deduced from this diminution of carotenoid content.

The maximum quantum yield of PS2 photochemistry assessed as F_v/F_m is currently much used for estimation of a stress impact on the photosynthetic apparatus. This parameter was depressed by the TMV, the values observed were below that found in healthy plants. Because this ratio serves as an indicator of photoinhibition or other kind of injury in response to various biotic and abiotic stresses (Gamon and Pearcy 1989, Groom and Baker 1992, Epron *et al*. 1995) we conclude that function of PS2 RC was damaged by the viral infection.

Similar conclusions were apparent from F_v/F_0 that can be used as a sensitive indicator of the maximum efficiency of photochemical processes in PS2 and/or the potential photosynthetic activity of healthy as well as stressed plants (Roháček 2002).

Further, the effective quantum yield of photochemical energy conversion in PS2, *i.e.* Φ_2, was also reduced by the TMV infection. This parameter reflecting actual performance of PS2 was more depressed in the first stage of infection than in the DGI in the second stage. This means that the DGI of the leaf perform photosynthesis with a better efficiency. On the other hand, the efficiency of PS2 in LGI with a higher content of the virus was greatly depressed.

NPQ was used to estimate the participation of several protective processes but mostly heat dissipation in the response of the leaf to a stress. The parameter q_N, a classical quenching ratio, could not be calculated from our data as we were not able to measure F_0' with our configuration. The non-photochemical energy dissipation was increased in TMV infected leaves. However, this process reached a maximum already in the first stage. As the infection progressed, this protection was no longer so pronounced. In DGI it was about the same as in healthy controls. In the second stage the elevated value of NPQ was observed in LGI but it was lower than in the first stage. This protective effect of the NPQ was reduced with a progression of the infection.

The photochemical activity of PS2 and especially of the whole electron transport chain in isolated chloroplasts declined very sharply in infected leaves. Comparing the results of Chl fluorescence kinetics and photochemical activity of PS2, we conclude that the more dramatic injury could be on the electron donor side of PS2, *i.e.* the oxygen evolving system. Also damage to the thylakoid components acting between PS2 and PS1 and in a domain close to PS1 probably occurred because substantial decrease of the rate of whole electron transport chain was observed in isolated chloroplasts of the infected leaves. The damage was more pronounced with progression of the infection and the highest injury was apparent in LGI.

The function of RCs of PS2 was impaired due to the disease. The type of alterations was not changed dramatically with depth of disease but the intensity did. Also other thylakoid constituents were damaged by the action of TMV. Depression of photosynthetic rate in TMV-infected leaves was related to progression of systemic mosaic symptoms. The LGIs in the second stage of infection with the highest content of virus were the most affected, whereas DGIs in several aspects were more similar to controls. With the onset of disease the plant initiated protective mechanisms related to non-photochemical quenching but they were diminished in a later stage of infection especially in DGIs.

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