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Mechanical wounding caused by inoculation influences the photosynthetic response of *Nicotiana benthamiana* plants to plum pox potyvirus

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Abstract

Plants of *Nicotiana benthamiana* (Gray) (60 d old) were mechanically inoculated by a spreading of the fourth and fifth leaves with inoculum with or without plum pox potyvirus (PPV). Changes in growth parameters and selected photosynthetic characteristics were followed in control and inoculated plants in the locally affected leaves (LA) during 11 d after inoculation (DAI), in systemically affected leaves immature at time of inoculation (SAI) during 14-25 DAI, and in systemically affected leaves developed after the inoculation (SAD) during 28-39 DAI. The pure mechanical damage caused by inoculation induced a decrease in the net photosynthetic rate (P_N) in LA and SAD leaves, and an increase in the steady-state value of the non-photochemical chlorophyll (Chl) fluorescence quenching q_N. The q_N increase appeared in certain time intervals in all measured leaves on plants, so it could be regarded as indication of a systemic reaction of plant to the local mechanical injury. The viral infection developed in LA leaves and spread to SAI and SAD leaves was documented by the ELISA-DASI method. The plant height and area of SAI and SAD leaves were lower in infected plants. The combined effect of mechanical damage and viral infection caused a decrease in P_N only in LA and SAD leaves. In SAD leaves, an increased relative height of the J step (V_J) in the O-J-I-P Chl fluorescence transient together with a lower B/A band ratio of thermoluminescence glow curves reflected a damage to the acceptor side of photosystem 2 (PS2) caused by the viral infection, and a faster kinetics of the induction of the photochemical quenching coefficient q_P of Chl fluorescence indicated a faster Q_A^- re-oxidation in the remaining undamaged centres of PS2.

Additional key words: chlorophyll fluorescence; CO_2 assimilation; ELISA-DASI; systemic response; net photosynthetic rate; thermoluminescence; tobacco; viral infection.

Introduction

Viral infection usually causes morphological and physiological alterations in the infected plants including changes in photosynthetic apparatus. Different changes in photosynthetic parameters may be found for different host plant-virus systems (Scholes 1992). Specific plant reaction to viral infection can be determined among others by whether virus or its components enter chloroplasts or not. For example, coat proteins of tobacco mosaic virus accumulate inside chloroplasts (Reinero and Beachy 1989) and even associate with photosystem 2 (PS2) complexes (Hodgson *et al.* 1989). An idea of viruses as chloroplast parasites appeared (Bedbrook *et al.* 1973, Daley 1995). The mechanism of direct viral action on PS2 remains unclear (see Balachandran *et al.* 1997). In plants infected with viruses whose components are detectable in chloroplasts, a decrease in the rate of CO_2 assimilation

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Abbreviations and symbols: C, control; Chl, chlorophyll; CP, coat protein; DAI, days after inoculation; F_{2ms}, Chl fluorescence intensity at 2 ms of the O-J-I-P transient; F_0, minimal Chl fluorescence intensity of the dark-adapted leaf; F_P, maximal Chl fluorescence intensity of the dark-adapted leaf measured by a PEA fluorometer; $F_V/F_P = (F_P - F_0)/F_P$, maximal quantum efficiency of photosystem 2 photochemistry; I, infected; LA, locally affected; MI, mock-inoculated; OD, optical density; P_N, net photosynthetic rate; PAR, photosynthetically active radiation; PPV, plum pox potyvirus; PS2, photosystem 2; Q_A, primary stable quinone acceptor of PS2; q_P and q_N, photochemical and non-photochemical quenching coefficients of Chl fluorescence, respectively; SAD, systemically affected leaves developed after inoculation; SAI, systemically affected leaves immature at time of inoculation; $V_J = (F_{2ms} - F_0)/(F_P - F_0)$, the relative height of the J step in the O-J-I-P Chl fluorescence transient.

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(Funayama *et al.* 1997) as well as a lower rate of electron transport through PS2 (Hodgson *et al.* 1989, Reinero and Beachy 1989) were found. *In vivo* chlorophyll (Chl) fluorescence is a sensitive indicator of the photosynthetic changes at thylakoid level (Krause and Weis 1991, Roháček and Barták 1999). Among firstly detected changes caused by viral infection belongs for instance an increase in non-photochemical quenching and decrease of photochemical efficiency of PS2, F_V/F_M (Baulcombe *et al.* 1995, Balachandran *et al.* 1997). Changes in Chl fluorescence (patchy quenching) may be detectable even before the appearance of visual symptoms (Osmond *et al.* 1990, Balachandran *et al.* 1994). The same holds for thermoluminescence detection (for review see Misra *et al.* 2001).

Some types of viruses probably do not enter chloroplasts and the chloroplast processes are influenced indirectly. It might be the case of PPV because its virions have been detected only in cytoplasm of infected cells, not in chloroplasts (Martin and Gélie 1997, Riedel *et al.* 1998). Indirect effect of such infection may result from metabolic changes in plant cells during establishment and replication of virus (Matthews 1991).

There is a spatial and temporal heterogeneity of functional and structural changes within the infected plant, among leaves in different ontogenetic stages at the time of inoculation. The directly inoculated leaves are mostly without visual symptoms even if they contain viral particles and the photosynthetic characteristics are changed (van Kooten *et al.* 1990, Rahoutei *et al.* 2000). The leaves expanding after inoculation are usually highly symptomatic. Different changes in Chl fluorescence parameters found in the asymptomatic and symptomatic leaves of tobacco plants infected by tobacco mosaic virus indicated different alterations in reaction centres of PS2 (van Kooten *et al.* 1990).

The usual inoculation procedure combines an effect of the viral introduction into cells with a necessary mechanical wounding of leaf surface tissue. Both the pathogen attack and wounding trigger a systemic response in plants although the signal pathways are probably different (Wildon *et al.* 1992, Sano and Ohashi 1995). The plant systemic reactions at the chloroplast level to mechanical wounding or viral infection may differ.

In this work we investigated an effect of PPV infection on photosynthetic characteristics of *Nicotiana benthamiana* (Gray) plants with regard to systemic reactions and accumulation of PPV within the plant. As mentioned above, PPV is thought not to enter chloroplasts so that only indirect and rather mild effects on chloroplast function might be expected. Therefore we concentrated also on the effect of mechanical damage caused by inoculation procedure with the aim to distinguish between reaction to pure mechanical stress (wounding) and to a combined effect of wounding and viral infection.

Materials and methods

Plants and virus: Seeds of *Nicotiana benthamiana* (Gray) were sown on January 18, 2001 into pots with perlite and grown hydroponically in a green-house, at temperature of 18±3 °C. On March 26, 2001 the plants were transferred to a growth chamber (8/16 h dark/light – "white light" of 50 μmol m^{-2} s^{-1}, 18/20 °C). The fourth and fifth leaves of chosen plants designated as infected (I) were inoculated with PPV-W isolate (*IPO*, Wageningen, the Netherlands) inoculum on the adaxial side (1 g of PPV-W systemically infected leaves was homogenised in 3 cm^3 of 0.01 M K_2HPO_4 supplemented by 30 mg of activated charcoal and 100 mg of celite). At the same time, the fourth and fifth leaves of other plants were inoculated with inoculum prepared in the same way from healthy *N. benthamiana* leaves [mock-inoculated (MI) plants]. Untreated plants were designated as control (C) plants. The measurements were done during the plant development within six weeks after the inoculation. The fourth and fifth leaves ("locally affected", LA) were measured during the first 11 d after inoculation (DAI). Further, two types of systemically affected leaves were measured: the leaves immature at time of the inoculation (SAI, the 7^{th}-9^{th} leaves on plant), and the leaves developed after the inoculation (SAD, the 10^{th}-13^{th} leaves on plant). SAI leaves were measured during 14-25 DAI, and SAD leaves during 28-39 DAI. A leaf area and height of plants were evaluated in several terms after inoculation.

Chl content was determined in 80 % acetone according to Lichtenthaler (1987) using a spectrophotometer *Lambda 40* (*Perkin-Elmer*, USA). The Chl content was related to the leaf area.

P_N was measured by an open gas exchange system *LCA-4* (*ADC BioScientific*, Hoddesdon, UK) on the attached *N. benthamiana* leaves after 10 min adaptation under leaf chamber conditions (PAR of 1 600 μmol m^{-2} s^{-1}, CO_2 concentration 500 μmol mol^{-1}). The measurements were performed on 11 DAI with LA leaves, on 25 DAI with SAI leaves, and on 39 DAI with SAD leaves of I, MI, and C plants.

Chl fluorescence measurements: The O-J-I-P Chl fluorescence transient was measured using a *PEA* fluorometer (*Hansatech*, King's Lynn, UK). The F_V/F_P ratio reflecting maximal quantum efficiency of PS2 photochemistry was determined as $(F_P - F_0)/F_P$, where F_P is the maximal Chl fluorescence intensity of the dark-adapted leaf measured by the *PEA* fluorometer, F_0 is the minimal Chl fluorescence intensity. The V_J parameter is defined as a ratio

$(F_{2ms} - F_0)/(F_P - F_0)$ (Strasser and Govindjee 1992), where F_{2ms} is the Chl fluorescence intensity at the time of 2 ms (in the region of the J step). The excitation irradiance was 4 300 μmol m^{-2} s^{-1} of PAR and time interval of detection was 2 s.

Induction kinetics of the photochemical (q_P) and non–photochemical (q_N) quenching of Chl fluorescence (Schreiber *et al.* 1986) were measured using a *PAM 2000* fluorometer (*Walz*, Effeltrich, Germany) during 280 s after switching on the actinic PAR of 200 μmol m^{-2} s^{-1}. The signal was collected from the adaxial side of dark-adapted (15 min) attached leaves at growth chamber conditions (20 °C). We evaluated both the steady-state values of q_P and q_N, and the values in the initial steeply increasing part of the induction curve (Matoušková *et al.* 1999). The most dynamic region of the induction of quenching coefficients for the case of *N. benthamiana* was at around 62 s. Only the most sensitive parameters, q_P (62 s) and q_N (steady-state at 280 s) were chosen for presentation. A general statistical description (medians and quartiles) was used in the case of all fluorescence parameters (Lazár and Nauš 1998).

ELISA-DASI procedure was performed according to Cambra *et al.* (1994). The microplates (*GAMA*, České Budějovice, Czech Republic) were coated with purified polyclonal IgG (rabbit anti-PPV-W, Palacký University, Olomouc, Czech Republic) in coating buffer (1 μg cm^{-3}). Samples prepared by homogenisation of leaves without petioles in the extraction buffer with the use of sand (1 g per 4 cm^3) were incubated overnight at 4 °C. The microplates with samples were incubated (2 h, 37 °C) with monoclonal antibodies (*Mabs*, Palacký University, Olomouc, Czech Republic) (1 mm^3 per 10 cm^3 conjugate buffer). The positive reaction was revealed by goat anti-mouse *IgG-AP* conjugate (*Sigma A2429*; 1 mm^3 per 5 cm^3 conjugate buffer, 2 h, 37 °C) and relative substrate. Optical density (OD, at 405 nm) of the microplate wells with samples against wells filled with buffer was measured after 1 h incubation at room temperature by means of a spectrophotometer *MRX 7000* (*Dynatech Laboratories*, Guernsey, UK). Dilution series of tested samples was used in order to obtain a relative content of viral coat protein (CP). We looked for such maximal dilution in which the measured sample was still positive (the samples with OD ≥ 0.2 were defined as positive). Several last points in a curve of OD dependence on dilution were fitted by a straight line, and an intersection of this line and a line of OD = 0.2 determined the value of maximal dilution.

Thermoluminescence glow curves were measured on 45 DAI using the laboratory set-up as was described by Skotnica *et al.* (1999). A leaf segment was linearly cooled from +20 °C to –65 °C at a cooling rate of 0.5 °C s^{-1} in darkness. Cooling was performed by contact of the sample holder with liquid nitrogen. Excitation of thermoluminescence was provided by continuous "white light" (50 μmol m^{-2} s^{-1}) from +20 °C to –50 °C, and signal was detected during heating of the sample at a rate of 0.47 °C s^{-1}. The measurements were performed in N_2 atmosphere in order to suppress the effect of chemiluminescence (Skotnica *et al.* 1999).

Non-denaturing *Deriphat*-PAGE of thylakoid pigment-protein complexes: Slight modification of procedures described by Peter and Thornber (1991a,b) and Peter *et al.* (1991) was used for non-denaturing *Deriphat*-PAGE separating Chl-containing protein complexes from thylakoids. Thylakoid membranes were isolated at 45 DAI from SAD leaves of C, I, and MI plants. Small leaf segments were mixed with chilled grinding buffer (0.4 M sorbitol, 10 mM Tricine-NaOH, pH 7.6, 10 mM $MgCl_2$) and homogenised three times for 5 s (400 rps) by a homogeniser *T 25 basic* (*IKA Labortechnik*, Staufen, Germany). The homogenate was filtred through 4 layers of *Miracloth* and centrifuged at 3 020×*g* for 2 min at 4 °C. The chloroplast pellet was re-suspended in 1 cm^3 of lysis buffer (25 mM Tricine-NaOH, pH 7.6, 2 mM Na_2EDTA) and the suspension was centrifuged at 22 000×*g* for 5 min at 4 °C. The obtained thylakoids were re-suspended and diluted to the final concentration 1 kg (Chl *a*+*b*) m^{-3} in the extraction buffer containing 11.3 mM Tris, 87 mM glycine, and 9 % (v/v) glycerol. The Chl *a*+*b* content was determined spectrophotometrically in 80 % acetone according to Lichtenthaler (1987).

Membrane solubilisation was carried out with 20 % decyl maltoside. This surfactant and membranes were mixed to yield a final 20 : 1 (m/m) ratio of surfactant to Chls. The surfactant extracts were centrifuged at 7 000×*g* for 2 min at 4 °C to remove the colourless insoluble material, and the green supernatant was immediately applied to PAGE. A polyacrylamide gel of 8.5 % [33.5 % (m/v) acrylamide, 0.3 % (m/v) bisacrylamide] containing 12.4 mM Tris, 48 mM glycine, pH 8.6, was polymerised with 0.1 % ammonium persulfate, Na_2SO_3 (1.5 mg per 1 cm^3 of gel) and 0.005 % TEMED. For the 3-mm thick gel a volume of 13 mm^3 of extract was loaded per line. The cooled reservoir buffer for electrophoresis was 12.4 mM Tris, 96 mM glycine, pH 8.3, and 0.2 % *Deriphat 160*. The electrophoresis was performed at 4 °C at 50 V constant voltage for 15 min and then at 90 V for 120 min (a device of *Bio-Rad*, USA) in darkness.

Results

Growth characteristics: The changes in growth characteristics during the 6 weeks of the experiment are shown in Tables 1 and 2. Initially (3-19 DAI) the C, I, and MI plants achieved a similar height, thereafter (25-39 DAI) the I plants grew slower in comparison with the C and MI plants. The mean height of the infected plants was on 39 DAI lower by about 20 % than that of control plants (Table 1). Similarly, the leaf area of the SAD and SAI leaves of infected plants was at 39 d after inoculation substantially lower (3 to 8 times) than the area of corresponding leaves of C and MI plants (Table 2). The growth parameters of the mechanically wounded plants (the MI plants) were only slightly lower than those of the control plants. All types of measured leaves on MI plants were slightly pale green.

In order to compare the functional reaction of leaves of different growth stages on the initial plant infection or mechanical wounding, the results obtained with individual types of leaves are described separately.

Table 1. Plant height of control and mock-inoculated plants of *Nicotiana benthamiana*, and plants infected by plum pox potyvirus (PPV) during 39 d after inoculation. Means and SD are shown, $n = 5$. Values within a row followed by different letters were significantly different at $p < 0.01^{**}$, $p < 0.05^{*}$.

DAI	Plant height [cm]		
	Control	Mock-inoculated	Infected
3	9.5±3.1	10.7±2.7	10.6±2.2
11	12.5±3.5	13.9±2.5	14.8±3.5
17	21.1±4.8	22.0±4.0	22.3±4.8
19	22.2±5.2	24.0±4.1	22.2±3.8
25	28.7±6.5	29.3±4.7	25.6±5.2
32	39.6±3.1a	37.8±4.7	31.6±4.7b*
39	47.8±2.3a	44.8±4.8a	35.8±5.4b**,*

Table 2. Leaf area of locally affected leaves (LA), and of systemically affected leaves immature at the time of inoculation (SAI) and developed after inoculation (SAD) of control and mock-inoculated plants of *Nicotiana benthamiana*, and plants infected by plum pox potyvirus during 39 d after inoculation. Means and SD, $n = 5$. Values within a row followed by different letters were significantly different at $p < 0.001^{***}$, $p < 0.050^{*}$.

Leaves	DAI	Leaf area [cm^2]		
		Control	Mock-inoculated	Infected
LA	11	4.2±1.0	3.5±0.8	3.8±0.9
	19	5.2±1.6	5.1±1.7	3.3±1.2
SAI	25	9.7±3.4a	8.7±2.0a	5.7±1.2b*
	39	12.4±3.2a	11.4±1.9a	3.7±1.4b***
SAD	25	8.5±2.4a	7.7±1.9a	1.2±0.4b***
	39	11.4±2.7a	9.7±1.7a	1.2±0.3b***

The locally affected (LA) leaves: No pronounced symptoms of infection were observed on LA leaves of infected plants, only solitary pale green spots on the leaf surface were detected from about 7 DAI. The number of these spots increased slightly with time after inoculation. Nevertheless, LA leaves of I plants exhibited senescence symptoms earlier than corresponding leaves of MI and C plants.

The mechanical injury of the inoculated (LA) leaves caused a decrease in P_N and in the Chl content *per* leaf area measured on the 11th d after inoculation (Fig. 1*A,C*). The combination of the mechanical injury and viral infection caused a more pronounced decrease in the rate of CO_2 assimilation *per* leaf area whereas the decrease in the Chl content was smaller.

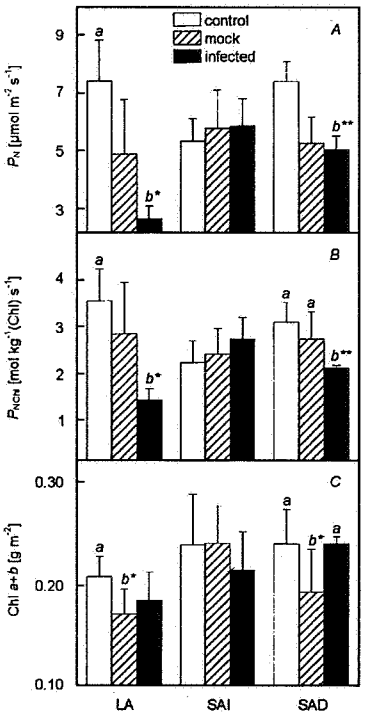

Fig. 1. Net photosynthetic rate *per* leaf area, P_N (*A*) and *per* chlorophyll content, P_{NChl} (*B*), and content of chlorophylls *a* and *b* (*C*) in locally (LA) and systemically affected (SAI, SAD) leaves of control, mock-inoculated, and infected with plum pox potyvirus *N. benthamiana* plants. Measurement was performed with LA leaves on the 11th, with SAI leaves on the 25th, and with SAD leaves on the 39th d after inoculation. Means and SD, $n = 5$. Different letters indicate statistically significant difference between columns at $p < 0.01^{**}$ or $p < 0.05^{*}$.

Minor changes in the F_V/F_P ratio (evaluated from the O-J-I-P transient) found in the LA leaves indicated that the photochemical efficiency of PS2 was not significantly affected by the mechanical injury of these leaves

(Fig. 2*A*). Similarly a profound change in the V_J parameter was not detected (Fig. 2*D*). A small decrease in F_V/F_P found on the 11th d after inoculation in the leaves of infected plants was statistically significant and may indicate a slight decrease of the photochemical efficiency of PS2 caused by the infection. The mechanical injury of leaves prevailed during the first 8 d after inoculation and caused a slowed off kinetics of q_P (Fig. 2*G*) and an increase of the steady-state q_N (Fig. 2*J*).

Accumulation of coat protein of PPV (PPV-CP) was detectable in the LA leaves from 5 DAI growing gradually and reaching maximum at about 21 DAI (Fig. 3). Pronounced senescence of these leaves was evident at 21 DAI.

The leaves immature at time of the inoculation (SAI): Visual symptoms of infection in SAI leaves were stronger than in LA leaves although the first symptoms were detected at the same time (round 7 DAI) for both types of leaves. At 7 DAI, the SAI leaves of I plants were gently deformed and the chlorotic mosaic was sporadically notable. These symptoms developed in the following days. Neither mechanical nor combined effects led to a decrease in the rate of CO_2 assimilation in the SAI leaves (Fig. 1*A*). The SAI leaves of the infected plants revealed even a slightly higher rate of photosynthesis on Chl basis than the ones of MI plants due to their lower Chl content (Fig. 1*B,C*).

In the SAI leaves which were not directly mechanically injured, the F_V/F_P was only slightly lower in the leaves of the MI and I plants than in the C-plants but the values above 0.83 (in most cases) indicated a good photochemical efficiency of PS2 (Fig. 2*D*). Similarly, no pronounced differences in V_J were found (Fig. 2*E*). Somewhat more pronounced differences were found in the quenching coefficients. The SAI leaves of the MI and I plants were characterised by increased values of q_P (62 s) (from the 14th till 23rd DAI) and of q_N (from the 23rd DAI) (Fig. 2*H,K*). A decline of q_P (62 s) on 25 DAI is the only discernible difference caused by the viral infection alone.

Presence of PPV-CP in SAI leaves of infected plants was high already at first measurement (14 DAI) with no clear further tendency. The high relative concentration of the PPV-CP remained until 39 DAI (Fig. 3).

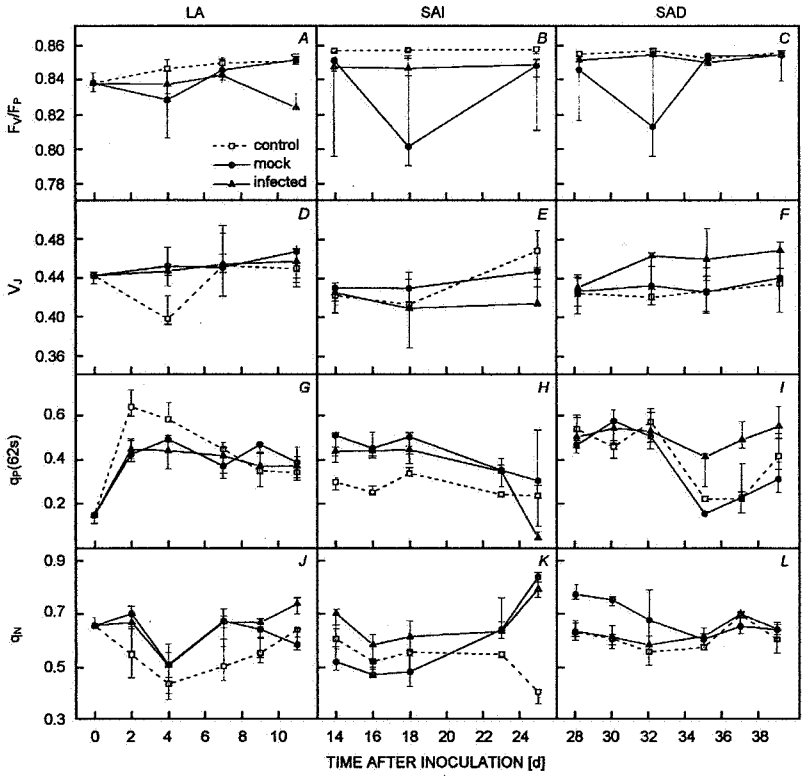

Fig. 2. Changes in the Chl fluorescence parameters F_V/F_P (*A, B, C*), V_J (*D, E, F*), q_P (62 s) (*G, H, I*), and q_N (steady-state; *J, K, L*) during 11 d after inoculation in the locally affected (LA) leaves (the 4th and 5th leaves, LA; *A, D, G, J*), during 14-25 d after inoculation in systemically affected leaves immature at the time of inoculation (SAI; *B, E, H, K*), and during 28-39 d after inoculation in systemically affected leaves developed after inoculation (SAD; *C, F, I, L*) of control and mock-inoculated plants of *N. benthamiana* and plants infected by plum pox potyvirus. Medians and quartiles, n = 5-8.

The leaves developed after the inoculation (SAD): Pronounced symptoms of viral infection were detectable already at the beginning of development of the SAD leaves. The leaves of I plants showed epinasty, their blades were markedly deformed (pustular surface and distortion). The leaves were generally pale green with

pronounced chlorotic mosaic, occasionally necrotic lesions were detected. The blade of SAD leaves of the MI plants had visually more homogeneous pale green colour in comparison with I plants although their Chl content was even lower (Fig. 1*C*).

The SAD leaves of the MI and I plants revealed a substantially lower rate of CO_2 assimilation *per* leaf area (Fig. 1*A*). The decrease in this rate was nearly the same in the leaves of both inoculated variants. However, the viral infection retarded the decrease in Chl content that was found only in the SAD leaves of the MI plants (Fig. 1*C*). Therefore, the photosynthetic rate on the Chl basis decreased in SAD-MI leaves less than in case of the SAD-I leaves (Fig. 1*B*).

The F_V/F_P was very similar in the SAD leaves of all plants including the 39th DAI (Fig. 2*C*). After 35 DAI the SAD leaves of C and MI plants behaved in a very similar way. However, the SAD leaves of the infected plants had higher values of V_J and q_P (62 s) (Fig. 2*F,I*). Similar increase of q_P (62 s) in younger parts of the infected plants was observed in an independent experiment (values not shown), therefore we suppose that the increased q_P (62 s) is a characteristic feature of those (symptomatic) parts of the infected plants. Significant changes of q_N were not found in the SAD leaves after 35 DAI (Fig. 2*L*).

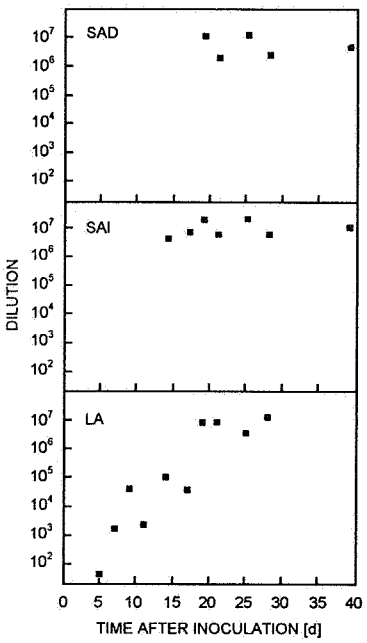

Fig. 3. Values of maximal dilution of samples prepared by ELISA-DASI procedure from leaves of *N. benthamiana* infected by plum pox potyvirus in which a measured sample was still positive (the samples with optical density ≥ 0.2 (at 405 nm) were defined as positive (see Materials and methods). The values of dilution reflect a relative content of viral coat protein in leaves. LA, locally affected leaves, measured during 5-28 DAI; SAI, systemically affected leaves immature at the time of inoculation measured during 14-39 DAI; SAD, systemically affected leaves developed after inoculation, measured during 19-39 DAI.

Relative concentration of PPV-CP in the SAD leaves of I plants was within the same range of values as that of the SAI leaves (Fig. 3): these values were highly variable.

The results of thermoluminescence measurements are shown in Fig. 4. The glow curves obtained under our conditions contained three different bands. The A-band around −10 °C can be ascribed to the $S_3Q_A^-$ recombination (Koike *et al.* 1986). The band at about 12 °C is probably the B-band ($S_{2/3}Q_B^-$ recombination, Rutherford *et al.* 1982). The band situated between 40 and 50 °C may be the C-band originating from the $Y_D^+Q_A^-$ recombi-

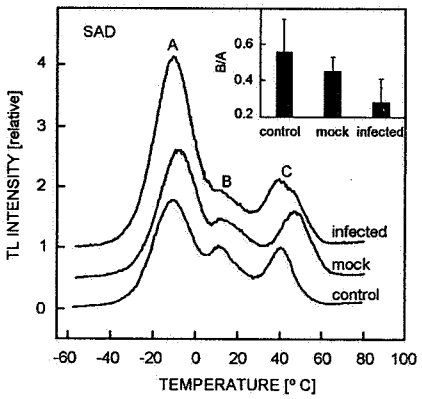

Fig. 4. Thermoluminescence glow curves with A, B, C bands of SAD leaves of control, mock-inoculated, and plum pox potyvirus infected *N. benthamiana* plants. Measured on the 45th d after inoculation, mean curves (n = 5). The vertical scale is the same for all curves, the curves are shifted vertically, their zero levels are shown on the right-hand abscissa. *Insert*: ratio of maximal thermoluminescence intensities in B and A bands of the glow curves. Means and SD, n = 5. *Different letters* indicate statistically significant difference between columns at $p < 0.05^*$.

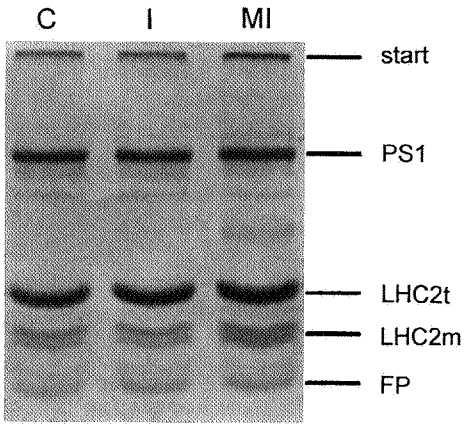

Fig. 5. Non-denaturing *Deriphat*-PAGE of thylakoid membranes isolated on the 45th d after inoculation from SAD leaves of control (C), plum pox potyvirus infected (I), and mock-inoculated (MI) *N. benthamiana* plants. The identities of the thylakoid pigment-protein complexes are marked on the right (Lee and Thornber 1995). PS1, photosystem 1; LHC2t, light-harvesting complex of PS2 (trimers); LHC2m, light-harvesting complex of PS2 (monomers); FP, free pigments.

nation (Demeter *et al.* 1993, Krieger *et al.* 1993) although some contribution of chemiluminescence can not be excluded (Skotnica *et al.* 1999). A simplified interpretation of the TL measurements may be based on a model in which the relative height of the B-band is proportional to the content of fully functional PS2 complexes. In this respect, the best situation of the SAD leaves was that of the control plants, a worse situation was in the MI plants, and the greatest relative decrease of the B-band was found for the I plants (Fig. 4, *insert*). The sequence of the variants corresponds to that observed in case of the rate of CO_2 assimilation based on Chl basis (Fig. 1*B*). The decrease of the B-band in the infected plants was accompanied by an increase of the band A. The C-band in the leaves of the MI plants was significantly shifted to higher temperature (Fig. 4). A reason for this shift has not been studied in this work.

The results of *Deriphat*-PAGE have shown no difference in the relative content of individual pigment-protein complexes of thylakoid membranes in SAI leaves of C and I plants of *N. benthamiana* (Fig. 5). In the leaves of MI plants, the electrophoretic bands containing light-harvesting complexes of PS2 (trimers and monomers) seemed to be slightly more pronounced than those of C or I plants.

Discussion

The infection of *N. benthamiana* plants by PPV may be characterised as a mild stress for the photosynthetic apparatus. This statement is based on the fact that the usual indicator of the stronger stress, a decrease of F_V/F_P under 0.8 was not observed in our case (see Fig. 2). One reason for a relatively mild effect is the indirect action of this virus on chloroplast metabolism due to the probable absence of viral particles inside chloroplasts of the infected cells (Martin and Gélie 1997, Riedel *et al.* 1998). We had to look for more gentle changes in the Chl fluorescence parameters similarly to the previous seek for the first Chl fluorescence indication of water stress (Matoušková *et al.* 1999). The main target of the infection seems to be localised in another metabolic and gene expression pathways.

The effect of mechanical injury: The pure mechanical injury of the older leaves of *N. benthamiana* plants at the age of 60 d had only a minor effect on the plant growth parameters during the following development (Tables 1 and 2). However, the photosynthetic characteristics revealed a delayed systemic reaction of the whole plant bearing a memory of the wounding in leaves directly unaffected (SAI) or even developing after wounding (SAD). Of special interest is the higher steady-state value of q_N developed in the injured (LA) leaves and appearing afterwards in the older (SAI) and younger (SAD) systemically affected leaves. The behaviour of non-photochemical quenching q_N resembles some kind of functional wave spreading along the plant from the older to younger parts due to the mechanical injury. Moreover, the young SAD leaves were deprived in general marks of photosynthesis, they had a lower rate of CO_2 assimilation and a lower content of Chls in comparison with the leaves of C plants.

A higher value of q_N evoked by a mechanical damage has been referred by Herde *et al.* (1999) for both directly wounded and unwounded leaves of tomato. In their case, the increase in steady-state q_N was detectable from 1 min to 5 h after wounding. We have shown a higher q_N after several days as a relatively long lasting systemic reaction. We suggest that the increase in q_N reported for virus-infected plants (*e.g.* van Kooten *et al.* 1990) may, at least in part, be induced by mechanical damage due to the inoculation procedure.

The effect of PPV infection: If we want to find the specific reaction of the plant to viral infection after mechanical inoculation, we have to exclude the wounding effect from the measured combined effect of wounding and infection. This is usually not done. The spreading of the virus within the infected plant has been documented by the presence of viral protein in the inoculated and lately also in the SAI and SAD leaves. In the leaves partly developed at time of inoculation (SAI), P_N both on leaf area and Chl basis did not decrease, rather an increasing tendency of this rate can be found despite of a relatively high content of viral CP. The content of the thylakoid pigment-protein complexes in SAD leaves seemed to be unchanged by the PPV infection.

However, the adverse effect of infection was manifested in the SAD leaves, *i.e.* in the newly accrued, highly symptomatic leaves. P_N was substantially lower with respect to the corresponding leaves of the control plants. The higher relative height of the J step in the O-J-I-P Chl fluorescence transient and a lower ratio B/A bands in the thermoluminescence glow curves indicated a higher relative amount of the PS2 centres damaged at the acceptor side. These are most probably the Q_B-non-reducing centres with inhibited electron transport from Q_A^- to Q_B (Melis 1991). The accumulation of the Q_B-non-reducing centres should retard the kinetics of the photochemical quenching q_P due to slowing down of the Q_A^- re-oxidation. However, the SAI leaves containing the virus proteins were characterised by the faster kinetics of the q_P rise indicating a more efficient linear electron transfer behind PS2, *i.e.* a faster re-oxidation of Q_A^-. The apparent contradiction could be explained by the faster Q_A^- re-oxidation in the remaining undamaged Q_B-reducing centres that represent the major population of the reaction centres of PS2 even in the highly symptomatic SAI leaves.

In *N. tabacum* infected with tobacco mosaic virus

(Reinero and Beachy 1989) and in *N. benthamiana* infected by pepper and paprika mild mottle viruses (Rahoutei *et al.* 1999, 2000), an inhibition of the donor side of PS2 (especially the inhibition of the oxygen evolving complex) has been suggested together with reduction in a fraction of open PS2 centres indicated by a decrease in steady-state photochemical quenching q_P (van Kooten *et al.* 1990, Rahoutei *et al.* 2000). However, these viruses probably enter chloroplasts so that their action on chloroplast function may be stronger and distinct from the action of PPV.

A more detailed study might be necessary to describe the whole systemic reaction of photosynthetic parameters to the local wounding and PPV infection of the *N. benthamiana* plants.

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