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Chlorophyll *a* fluorescence as a tool for a study of the *Potato virus Y* effects on photosynthesis of nontransgenic and transgenic P*ssu-ipt* tobacco

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Abstract

The effect of *Potato virus* Y^{NTN} (PVY) infection upon photosynthesis was analysed in transgenic P*ssu-ipt* tobacco overproducing endogenous cytokinins in comparison with control, nontransgenic *Nicotiana tabacum* plants. The course of the infection from the early to the late stage was monitored by measuring of photosynthetic gas exchange and fast chlorophyll (Chl) *a* fluorescence induction kinetics. Leaf photosynthesis was also analysed using Chl fluorescence imaging (Chl-FI). From the different fluorescence parameters obtained using Chl-FI, the nonphotochemical quenching (NPQ) proved to be the most useful parameter to assess the effect of PVY infection. On the other hand, Chl-FI was found to be inapplicable for any presymptomatic detection of PVY infection in tobacco. The lower accumulation of the virus was found in transgenic plants and corresponded also with the presence of visible symptoms of PVY infection. The net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) significantly decreased with the progress of the infection in both control plant types and transgenic rooted plants, while transgenic grafts were much less affected. The analysis of the Chl fluorescence transient revealed higher number of silent dissipative reaction centres, higher nonphotochemical dissipation, and significantly lower performance index, $PI_{(abs)}$, in the healthy transgenic grafts. Chl-FI also confirmed significantly higher NPQ in transgenic grafts.

Additional key words: chlorophyll *a* fluorescence imaging; cytokinins; gas-exchange parameters; *ipt*; photosynthesis; *Potato virus Y*; transgenic tobacco.

Introduction

Viruses cause many important plant diseases and they are responsible for huge losses in crop production and quality all over the world. *Potato virus Y* (PVY) belongs to the largest plant virus family, *Potyviridae*. PVY^{NTN} isolate, used in this study, produces superficial necrotic ring spots on infected potato tubers; beside this, it has also an ability to infect tobacco systemically. In tobacco, PVY^{NTN} causes a leaf distortion and a severe vein necrosis in leaves and stems eventually leading to a death of the infected plant.

Whereas many studies have been directed towards understanding the structure, genetics, and a transport of viruses in plants, much less is known about the impact of a virus infection on plant physiology and about mechanisms of a virus resistance/tolerance in plants. Many studies reported that viral infections lead in plants to changes in their metabolism and to alterations in their photosynthetic capacity. However, general mechanisms that lead to metabolic perturbations and symptom

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Abbreviations: C – nontransgenic rooted plants; C/C – nontransgenic grafts; Chl – chlorophyll; Chl-FI – Chl fluorescence imaging; CK – cytokinin; DAI – days after the inoculation; E – transpiration rate; F_m – the maximal fluorescence in the dark-adapted state; F_m'– the maximal fluorescence in the light-adapted state; F_v/F_0 – the ratio of efficiency of electron donation to PSII; F_0 – the fluorescence intensity at 50 μs; g_s – stomatal conductance; *ipt* – the isopentenyl transferase gene; NPQ – nonphotochemical quenching; $PI_{(abs)}$ – performance index; P_N – net photosynthetic rate; P*ssu-ipt* – the isopentenyl transferase gene under the control of the light-inducible promoter for the *Pisum sativum* small subunit of Rubisco; PSII – photosystem II; PVY – *Potato virus* Y^{NTN}; ROI – a region of interest; T – P*ssu-ipt* transgenic rooted plants; T/C – P*ssu-ipt* grafts grown on nontransgenic rootstock.
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development following the viral infection are poorly understood, partially because the infectious process and its physiological consequences are quite variable (Balachandran *et al.* 1997). It is known that the onset of leaf symptoms caused by plant viruses in their hosts depends on changes in the chloroplast structure and function. Viral infections often decrease the rate of photosynthesis, particularly due to stomata closure, and a reduction of electron transport and the inhibition of photosystem II (PSII) were also often found (Zhou *et al.* 2004, Guo *et al.* 2005, Funayama-Noguchi and Terashima 2006, Song *et al*. 2009).

Fast Chl fluorescence induction kinetics is defined as a Chl fluorescence transient recorded on a dark-adapted plant during the first few seconds after the exposition to the very short (1–2 s) actinic radiation (Roháček 2002, Roháček *et al*. 2008). The analysis of the transient with a typical polyphasic shape exhibiting several distinguishable Chl fluorescence levels (O, J, I, P) can offer detailed information on the structure and function of plant photosynthetic apparatus, particularly of PSII (Strasser and Strasser 1995, Strasser *et al.* 2000, 2004).

Chl fluorescence imaging (Chl-FI) enables to measure the distribution of Chl fluorescence emission in two dimensions. Chl-FI has proved to be a valuable and non-destructive tool to study the spatial and temporal heterogeneity of the leaf photosynthesis under a biotic stress even before the manifestation of visible symptoms (Chaerle *et al.* 2004, Pérez-Bueno *et al.* 2006).

A viral infection also modulates hormone levels in plants (for a review *see* Jameson and Clarke 2002). Concerning cytokinins (CK), alterations in their metabolism, indicating the virus influence on their biosynthesis, catabolism, and/or conjugation, were found in different host-virus combinations. Several studies on virus-infected plants found metabolism of CK significantly altered in favour of conjugation and reduction of active forms (Dermastia *et al.* 1995, Zhang *et al*. 1997, Clarke *et al*. 1999). Several reports also demonstrated that enhanced tolerance to the viral infection may be related to elevated CK content (Clarke *et al.* 2000, Pogány *et al.* 2004). However, experiments studying the influence of externally applied CK on virus-induced necrotic symptoms and related metabolic changes brought contradictory results depending on the timing, a form of the application, and the applied concentration (Jameson and Clarke 2002).

Experiments with transgenic tobacco harboring the isopentenyl transferase (*ipt)* gene for the enzyme that catalyses the rate-limiting step in CK biosynthesis, proved that endogenous CK overproduction affects the plant growth, development, senescence, and also the resistance to abiotic and biotic stresses (Synková and Valcke 1999, Synková *et al*. 2006a,b). In our previous work, we studied the effect of PVY infection upon the photosynthesis of P*ssu-ipt* tobacco using gas-exchange and Chl fluorescence quenching measurements particularly in the late stage of the infection, when the visible symptoms already occurred. We have shown that despite a lower photosynthesis prior PVY inoculation, the P*ssu-ipt* tobacco plants were less affected by PVY infection (Synková *et al.* 2006a).

The Chl-FI system (Valcke *et al*. 1999, Ciscato 2000) and fast Chl fluorescence induction kinetics used in this study allowed to gain a further insight into the processes induced by PVY infection in photosynthetic apparatus. We followed the course of the infection from the early to the late stage focusing also on a possibility of pre-symptomatic detection of PVY infection. In this study, we combined measurements of fast Chl fluorescence induction kinetics using *Plant Efficiency Analyzer (PEA*, *Hansatech Instruments Ltd.*, UK) and Chl fluorescence imaging system (Chl-FI) to obtain more complex picture of the influence of the virus infection on photosynthesis in the transgenic P*ssu-ipt* tobacco plants.

Materials and methods

Plant material: Control, nontransgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was grown as rooted plants (C) from seeds. Transgenic tobacco, containing a supplementary isopentenyl transferase gene under the control of the light-inducible promoter for the *Pisum sativum* small subunit of Rubisco (P*ssu-ipt*), was generated by means of the *Agrobacterium tumefaciens* transformation system and grown *in vitro* as shoots unable to form roots (Beinsberger *et al.* 1991). The transgenic shoots were grafted onto control tobacco rootstock and grown as grafts (T/C). P*ssu-ipt* transgenic rooted plants (T), *i.e.* the first autogamic progeny of the transgenic grafts, which are able to form a small root system, were grown from seeds, selected *in vitro* on an agar medium with kanamycin and then transferred into soil. Control grafts (C/C) were made from C shoots grown *in vitro*, grafted onto C rootstock. All plants were grown after *in vitro* precultivation in pots with soil substrate in a greenhouse, under temperatures of 25ºC day/18ºC night and 60% relative humidity. For *Potato virus Y* (PVY) inoculation, the plants in the early vegetative stage were used (C – 8 weeks old, T –12 weeks old, C/C – 3 weeks after grafting, T/C – 5 weeks after grafting). Although the absolute age of control type and both transgenic types was different, the samples were taken from the plants in the comparable stages of the plant development. All measurements were done at the level of the first fully developed leaf in intervals of 3 (the early stage of the infection), 7 (the middle stage of the infection), and 14 (the late stage of the infection) days after the inoculation (DAI) with PVY.

Inoculation of plants with PVY^{NTN} isolate: Plants in the early vegetative stage were mechanically inoculated with virus isolate of PVY^{NTN} (provided by Ing. Petr Dědič, CSc., Institute of Potato Research, Havlíčkův Brod, Czech Republic). The lower mature leaves dusted with carborundum powder were rubbed with a suspension of virus-infected tissue in 0.057 M K_2HPO_4, pH 8.0.

DAS-ELISA: Leaf samples were frozen in liquid N_2 and stored at –75°C. Virus content was determined in homogenates of the leaves of healthy and infected plants by DAS-ELISA at 450 nm (Clark and Adams 1977) using polyclonal antibodies against the PVY (Čeřovská 1998).

Gas-exchange measurements: Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were measured using portable infrared gas analyzer *LCA-4* (*ADC BioScientific Ltd*., UK). Measurements were conducted on attached leaves under the irradiance of 600 μmol m^{-2} s^{-1}, temperature of 25°C, CO_2 concentration of 380 μmol mol^{-1} and 30% air humidity after 30-min acclimation to such condition. Beside the leaf and stages mentioned above, gas exchange measurements were also conducted on the leaf above the originally measured one that became mature during the course of the experiment (14 DAI).

Visualization of NPQ on the leaf surface using fluorescence imaging system: In this study, the fully computer-controlled Chl fluorescence imaging system (Chl-FI) described by Valcke *et al.* (1999) and Ciscato (2000) was used. After 30-min dark adaptation, a plant was placed into the instrument cabinet with the leaf fixed in a horizontal position. A saturating pulse (1,200 μmol m^{-2} s^{-1}, λ < 650 nm) lasting 1 s was applied at time intervals 0, 2, 5, 10, 20, 30, 60, 90, 120, 180, 240, and 300 s (12 pulses) after the onset of continuous actinic illumination (200 μmol m^{-2} s^{-1}, λ < 650 nm). The instrument control and data collection was carried out under *Linux*, distribution *Slackware 7.0*, *Kernel version 2.0.13*. The software for recording and processing the images was described in Ciscato (2000). The image processing (dark signal subtraction and intensity correction) was done *via* scripts for the (free) student version of the *KhorosPro2001 suite* (*Khoral Research Inc*., Albuquerque, NM, USA).

To visualize NPQ pattern on the leaf surface, a region of interest (ROI) of 400 × 300 pixels representing the middle region of the right half of the tobacco leaf (viewed from the petiole) was selected for each series of corrected images. The NPQ was calculated for each of 12 excitation pulses as $\Delta F_m/F_m' = (F_m - F_m')/F_m'$ (Scholes and Rolfe 1996) by computing pixel by pixel. As an additional step in the image analysis, ROIs of 10 × 10 pixels in the most contrasting areas adjacent to the leaf veins were selected for each series of the corrected images to follow the time course of the fluorescence quenching with only a marginal influence of varying surface properties.

Chl *a* fluorescence kinetics: Fast Chl *a* fluorescence induction kinetics was measured using *Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd.,* UK). Chl fluorescence was excited with an excitation light of six red light emitting diodes (peak λ 650 nm; 1,200 μmol m^{-2} s^{-1} PAR) and detected by a PIN photodiode after passing through a long pass filter. Chl *a* fluorescence kinetics was measured on the first mature upper leaf 3, 7, and 14 DAI. After 30 min of leaf dark acclimation, Chl fluorescence transients were recorded for 1 s with data acquisition rate of 10 μs for the first 2 ms and 1 ms for the rest of the time. The measured points of the polyphasic rise of the fluorescence transient, *i.e.* F_0 (50 μs), F_J (2 ms), F_I (30 ms), and F_m were analyzed according to the JIP-test of Strasser and Strasser (1995) and Strasser *et al.* (2000, 2004). The parameters selected for this study are presented in Table 1.

Statistical analysis: Gas-exchange measurements and Chl-FI were carried out with five plants of each plant type cultivated and inoculated in three independent series. Parameters of Chl *a* fluorescence kinetics were measured on five plants in two independent series, two measurements were conducted on each inspected leaf. Statistically significant differences in the mean values were tested by *ANOVA* or *t*-test at $p = 0.05$ using *Sigmaplot 11.0*.

Results and discussion

Viral infection: The relative virus content during the infection was in a correlation with observed symptoms of the infection, *i.e.* the vein and stem necrosis and chlorosis, similarly as it was found in our previous experiments (Ryšlavá *et al.* 2003, Synková *et al*. 2006a, Doubnerová *et al*. 2009). C and C/C plants always showed visible symptoms of the infection, the first symptoms started appearing *ca*. 7 DAI (Fig. 1). The process of the infection was slightly delayed in T, but finally all plants showed the symptoms of the infection. No symptoms were observed in T/C. The relative virus content increased significantly in all infected control plants (C: 1.9 ± 0.2 and C/C: 1.7 ± 0.1) during 14 DAI. In T, the virus accumulated less (0.7 ± 0.1) than in non-transgenic controls but still more than in T/C (0.4 ± 0.1).

The lower relative virus content observed in both transgenic plant types was in accordance with the observation of Pogány *et al*. (2004) who found the

Table 1. Explanation of the selected chlorophyll fluorescence parameters.

Parameter	Explanation	Formula
F_0	fluorescence intensity at 50 μs	
F_m	maximal fluorescence intensity	
F_v/F_0	reflects the efficiency of electron donation to the PSII RCs and the rate of photosynthetic quantum conversion at PSII RCs	$(F_m - F_0)/F_0$
V_J	normalized variable Chl fluorescence at the J-step of the fluorescent transient	$(F_{2ms} - F_0)/(F_m - F_0)$
V_I	normalized variable Chl fluorescence at the I-step of the fluorescent transient	$(F_{30ms} - F_0)/(F_m - F_0)$
φ_{P0}	maximum quantum yield of primary photochemistry	$1 - (F_0/F_m) = F_v/F_m$
ψ_0	expresses the probability that a trapped exciton moves an electron into the electron transport chain further than Q_A^-	$1 - V_J$
φ_{E0}	quantum yield of electron transport	$\varphi_{P0}\,\psi_0 = (1 - F_0/F_m)(1 - V_J)$
φ_{D0}	quantum yield of energy dissipation	F_0/F_m
ABS/RC	total absorption capacity per photochemically active RC	$(TR_0/RC)/\varphi_{P0} = (M_0/V_J)/(1 - F_0/F_m)$
TR_0/RC	the maximal trapping rate of PSII, expresses the rate by which an exciton is trapped by the RC	M_0/V_J
DI_0/RC	ratio of the total dissipation of untrapped excitation energy from all RCs with respect to the number of active RCs	$(ABS/RC) - (TR_0/RC)$
RC/CS_0	density of photochemically active PSII RCs per cross section	$F_0\,\varphi_{P0}\,(V_J/M_0)$
$PI_{(abs)}$	performance index	$[(1 - F_0/F_m)/(M_0/V_J)]\,[(F_m - F_0)/F_0][(1 - V_J)/V_J] =$ $= [RC/ABS][\varphi_{P0}/(1 - \varphi_{P0})][\psi_0/(1 - \psi_0)]$
M_0	net rate of PSII closure, expresses the net rate of the closure of RCs	$(TR_0/RC) - (ET_0/RC) = 4\,(F_{300\mu s} - F_0)/(F_m - F_0)$

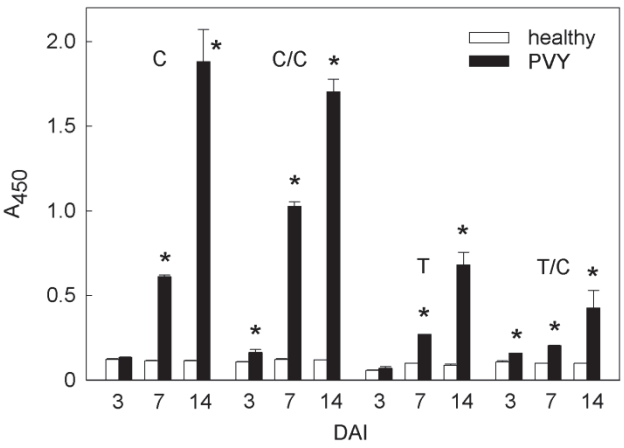

Fig. 1. The relative virus content in the leaves of healthy (*open columns*) and PVY infected (*closed columns*) control rooted plants (C), control grafts (C/C), transgenic rooted plants (T), and transgenic grafts (T/C) 3, 7, and 14 days after the inoculation (DAI). The values are means ± SE. Statistically significant differences between healthy and PVY infected plants are marked by an asterisk (P<0.05).

inhibition of virus-induced necrotic symptoms as well as a lower content of viral coat protein in CK overproducing tobacco line (CTK_m) transformed with a *CaMV 35S:ipt* gene infected with *Tobacco necrosis virus* (TNV). They attributed this to a higher antioxidant capacity, namely to increased ascorbate peroxidase and catalase activities and to higher ascorbate contents, found in the CK overproducing tobacco. This is also in agreement with our previous study, where the overproduction of CK in P*ssu-ipt* tobacco resulted in the stimulation of activities of antioxidant enzymes throughout the plant ontogeny (Synková *et al*. 2006b).

Photosynthesis and gas exchange: The statistical analysis of gas-exchange parameters (P_N, E, and g_s) proved the significant dependence on the plant type, PVY infection, and the stage of the infection (DAI) (Figs. 2,3). Both transgenic (T, T/C) and grafted (C/C) showed lower rates of P_N compared with healthy C. Healthy C/C and T reached *ca*. 75% and T/C *ca*. 50% of the values found in C. The significant decline in P_N of healthy C/C during the course of the experiment corresponded with their apparently faster plant senescence, which was observed compared with C. Both transgenic types, namely T/C, also exhibited lower E and g_s compared with C.

In the early stage of the virus infection (3 DAI), photosynthesis and plant water regime was not

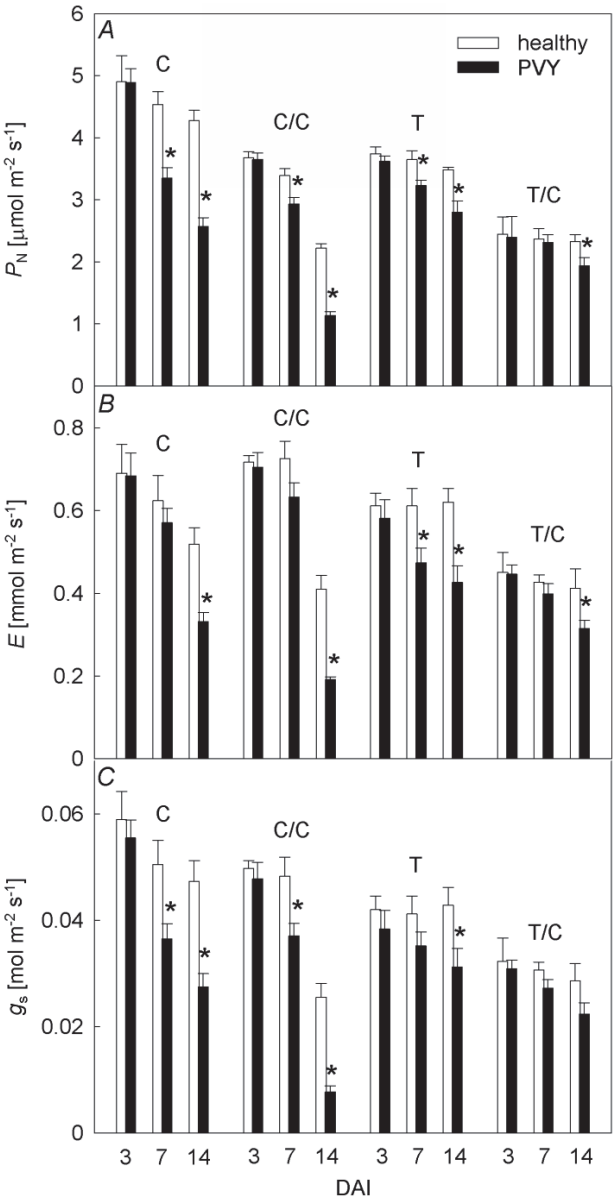

Fig. 2. Gas-exchange parameters in healthy (*open columns*) and PVY infected (*closed columns*) control rooted plants (C), control grafts (C/C), transgenic rooted plants (T), and transgenic grafts (T/C) 3, 7, and 14 days after the inoculation (DAI). (*A*) net photosynthetic rate (P_N); (*B*) transpiration rate (E); (*C*) stomatal conductance (g_s). The values are means ± SE. Statistically significant differences between healthy and PVY infected plants are marked by an asterisk (P<0.05).

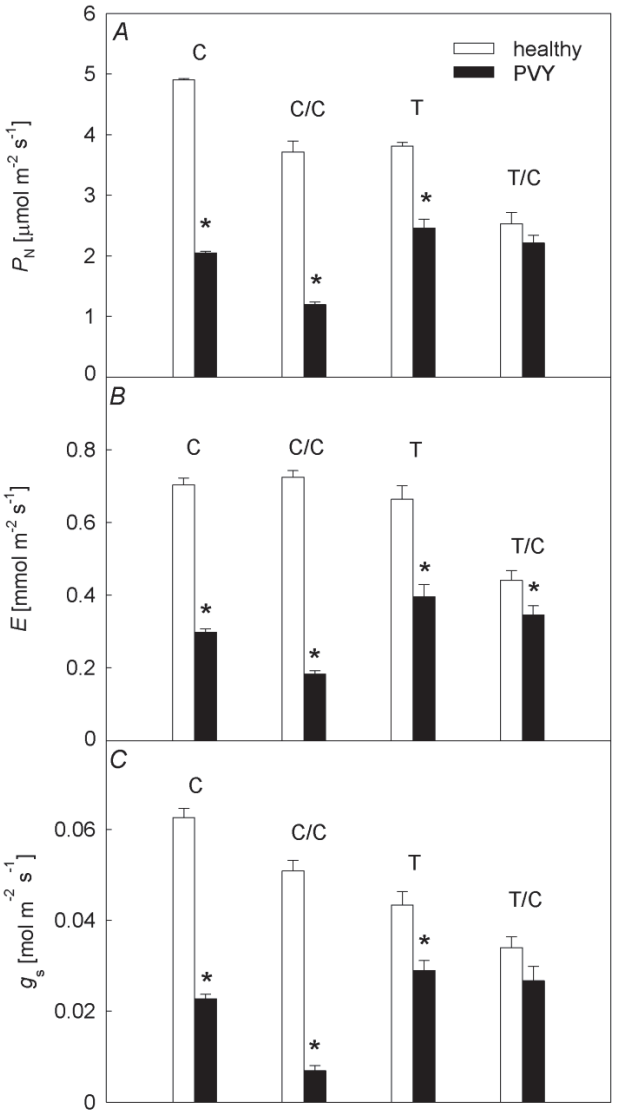

Fig. 3. Gas-exchange parameters in upper leaves of healthy (*open columns*) and PVY infected (*closed columns*) control rooted plants (C), control grafts (C/C), transgenic rooted plants (T), and transgenic grafts (T/C) 14 days after the inoculation (DAI). (*A*) net photosynthetic rate (P_N); (*B*) transpiration rate (E); (*C*) stomatal conductance (g_s). The values are means ± SE. Statistically significant differences between healthy and PVY infected plants are marked by an asterisk (P<0.05).

significantly influenced by PVY in any plant type (Fig. 2). In the middle stage of the infection (7 DAI), P_N decreased significantly in all plant types except T/C. The decline was more pronounced in both control plant types, where PVY infected C declined to 73% and C/C to 80% of the values in respective healthy plants, while T still kept *ca*. 90% of the respective control. PVY infection of 7 DAI led in all examined plants to a slight decrease of E, however, it was statistically significant only in T. The g_s decreased markedly in both control plant types, whereas the decrease in T and T/C was insignificant. The similar trends were visible in the late stage of the infection (14 DAI). PVY infection caused significant decrease in P_N, E, and g_s in all plant types, particularly in both control types (P_N to 60% in C and to 50% in C/C compared with respective healthy plants) than in transgenic (P_N to 75% in T, and to 83% in T/C, compared with respective healthy plants) types. The influence of PVY was even more pronounced in the upper leaves, which were still growing during the course of the infection, where P_N declined to 42% in C, 32% in C/C,

and 64% in T (Fig. 3). The moderate decrease of P_N and changes in g_s in T/C were not statistically significant. The most severe decrease of g_s (to 14% of healthy plants) was found in C/C.

Our present results showed that the photosynthesis declined with the increasing accumulation of PVY proteins in the infected plants, namely in both control plant types (C, C/C). This decline could be caused also by a decrease in g_s, which was observed during PVY infection. Thus the stomatal limitation of CO_2 influx to photosynthesis could be one of the major factors. Although contradictory data can be found in the literature (Arias *et al.* 2003), our previous experiments with PVY infected tobacco proved the stomata closure at the stage, when severe symptoms of the infection were observed (Synková *et al.* 2006a). The reason for this virus effect is not quite clear; however, electron microscopic pictures of infected plants show that stomata guarding cells were overloaded by viral protein aggregates, even more than other leaf cells. If this influenced somehow their function, it is beyond this study; nevertheless, it could surely affect their proper functioning. This could also explain the reason, why no effect on the photosynthesis and gas exchange was observed before the massive virus accumulation occurred, *i.e*. in the earlier stages of the infection. T/C plants showed the marginal virus content even in the late stage of the infection; therefore no significant changes in stomata functioning were observed. Nevertheless, beside of the stomatal limitation of P_N, our previous study proved also lower Rubisco activity in the late stage of PVY infection (Ryšlavá *et al*. 2003). Therefore also nonstomatal limitation should be taken into account.

Fast Chl *a* fluorescence induction kinetics: The thorough analysis of various parameters of a fast Chl *a* fluorescence induction kinetics proved that PVY infection affected primary photochemistry and functioning of PSII centres less than the plant type itself (Fig. 4, Table 1S – *see* the supplementary material online). Generally, the only significant differences between the healthy and infected plants were found in the late stage of PVY infection (Fig. 4*C*), while namely transgenic T/C showed differences in primary photochemistry and electron transport parameters compared with nontransgenic C throughout the whole experiment. T/C showed lower values of F_m, lower values of the ratio of F_v/F_0, higher values of variable Chl fluorescence at the J-step (V_J), the highest total absorption capacity per photochemically active reaction centre (ABS/RC), the highest maximal trapping rate of PSII (TR_0/RC), the highest values of the ratio of DI_0/RC, higher net rate of PSII closure (M_0), lower values of probability that a trapped exciton moves an electron further than to Q_A^- (ψ_0), lower maximum yield of electron transport (φ_{E0}), the lowest density of photochemically active PSII centres (RC/CS_0), and the lowest performance index ($PI_{(abs)}$), compared with other plant types.

A possible explanation for the lower values of F_0 found in T/C could be a higher proportion of photochemically inactive PSII reaction centres (*i.e.* non-Q_B-reducing or silent reaction centres), which may act as dissipative sinks, thereby decreasing F_0 (Öquist *et al.* 1992). In our previous study, Chl fluorescence emission spectra at 77 K indicated the presence of aggregates of light-harvesting complex proteins (LHCII) that were not connected to reaction centres of PSII in P*ssu-ipt* transgenic tobacco (Synková *et al.* 2005). Lower F_m is again an indication of the energy dissipation *via* nonphotochemical pathways. The lower F_v/F_0 found in T/C reflected here the lower relative contribution of the trapping flux to the total deexcitation fluxes of excited Chl (Strasser *et al*. 2000), which also suggests the presence of protection mechanisms such as nonphotochemical energy dissipation. In our present experiment, the reduced efficiency of the electron transport due to photochemically inactive reaction centres was reflected in higher V_J in T/C representing the fraction of closed reaction centres at 2 ms (Strasser and Strasser 1995, Strasser *et al*. 2000). A significantly higher ABS/RC, total absorption capacity per photochemically active reaction centre, indicated an apparent increase of the relative antenna size by the formation of silent reaction centres (Strasser *et al*. 2004). The highest TR_0/RC, the maximal trapping rate of PSII, was again an indication of the downregulation of PSII by forming silent reaction centres (Strasser *et al*. 2004). Due to the the higher number of silent dissipative reaction centres, the nonphotochemical dissipation increased in T/C, which resulted in a high DI_0/RC. Because of the presence of a high number of silent reaction centres, the density of the photochemically active reaction centres per cross section, RC/CS_0, was low in T/C. The performance index, $PI_{(abs)}$ characterizing plant vitality was significantly lower in T/C.

The only significant changes between the healthy and PVY infected plants were found in the late stage of the infection (14 DAI, Fig. 4*C*) in C, C/C, and T plants. In T/C, PVY infection had no significant effect on any of the examined parameters even 14 DAI. PVY infection shifted the values of the parameters in the same way (increased or decreased) in all plant types but only some of the changes were significant. PVY infection significantly increased F_0 in C and C/C and decreased the F_v/F_0 ratio in C and T. PVY infection caused significant increase in φ_{D0} in T plants. ABS/RC markedly declined in PVY infected C/C. PVY infection increased M_0 in C and C/C. DI_0/RC increased significantly in PVY infected C, C/C, and T plants, while $PI_{(abs)}$ decreased there.

Generally, PVY infection negatively influenced the function of PSII, with a tendency to increase F_0, to decrease F_m, and the F_v/F_0 ratio. The F_v/F_0 ratio reflects the efficiency of electron donation to the PSII reaction centres and the rate of photosynthetic quantum conversion at PSII reaction centres. The declining F_v/F_0 ratio caused by the PVY infection therefore reflected

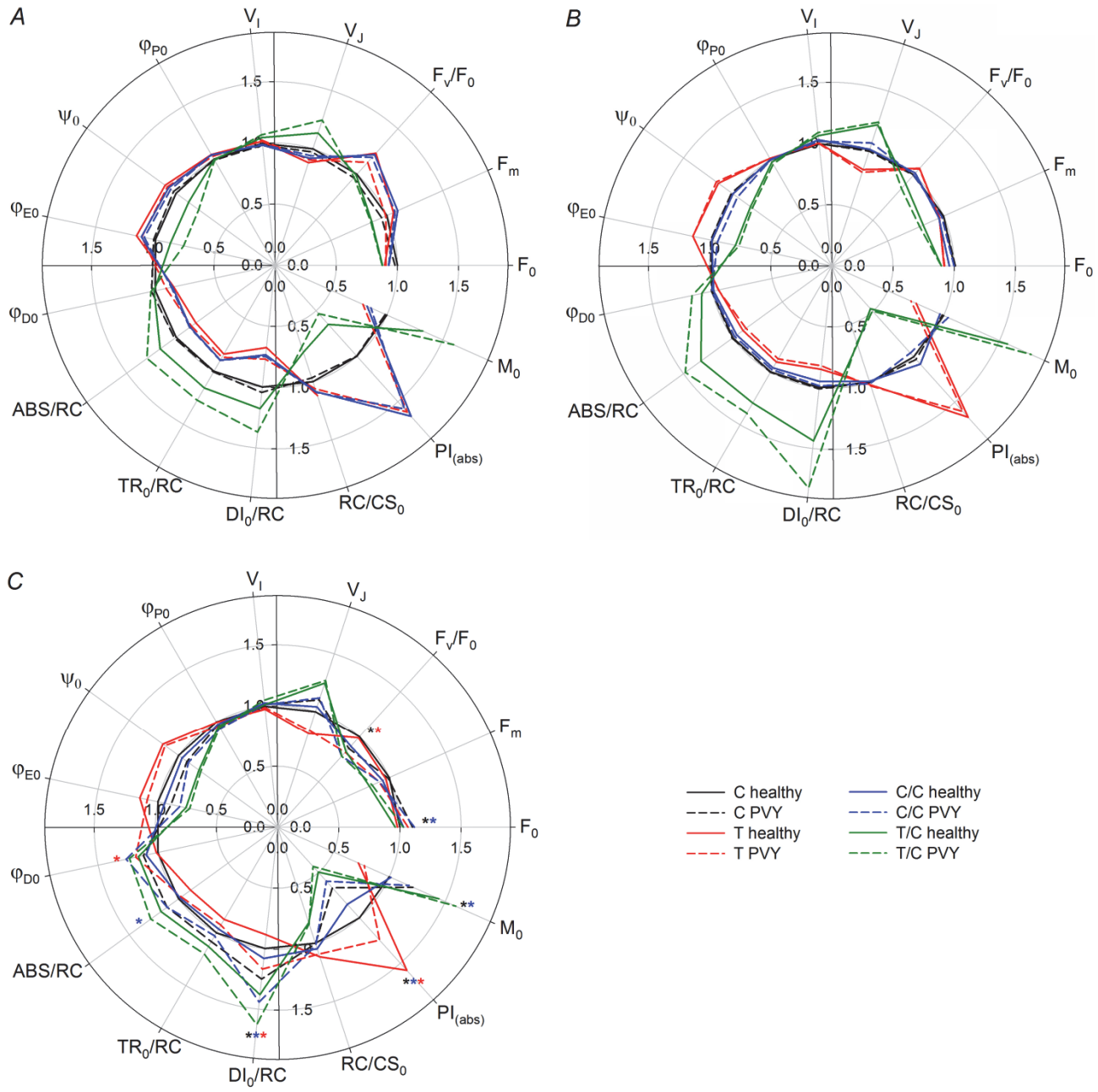

Fig. 4. The measured and calculated chlorophyll fluorescence variables relative to the healthy control rooted plants (C) in respective stage of the infection in healthy (*full black line*) and PVY infected (*dashed black line*) control rooted plants (C), healthy (*full blue line*) and PVY infected (*dashed blue line*) control grafts – C/C, healthy (*full red line*) and PVY infected (*dashed red line*) transgenic rooted plants - T, and healthy (*full green line*) and PVY infected (*dashed green line*) transgenic grafts – T/C in the early (*A*), middle (*B*), and late (*C*) stage of the infection. For the meaning of the parameters see Material and Methods section. Statistically significant differences between healthy and PVY infected plants are marked by an asterisk in respective colour (P<0.05).

a decrease in the relative contribution of the trapping flux to the total deexcitation fluxes of excited Chl (Strasser *et al*. 2000). It was in a connection with the increased nonphotochemical dissipation (DI_0/RC) found in PVY infected plants.

Plant vitality could be characterized by performance index, $PI_{(abs)}$ (Strasser *et al*. 2000). $PI_{(abs)}$ combines the three main functional steps (light energy absorption, excitation energy trapping, and conversion of excitation energy to electron transport) of photosynthetic activity by a PSII reaction centre complex into a single multi-parametric expression (for exact formula *see* Materials and methods). $PI_{(abs)}$ gives quantitative information on the current state of plant performance under stress

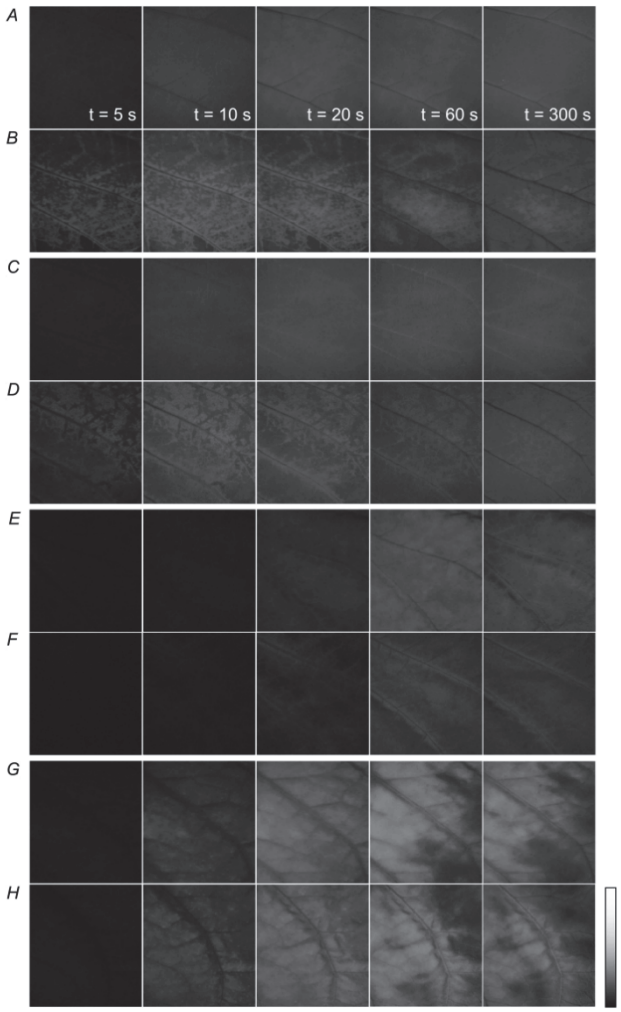

Fig. 5. Images of nonphotochemical quenching kinetics during fluorescence induction (at 5, 10, 20, 60, and 300 s) from healthy (*A*) and PVY infected (*B*) control rooted plants - C, healthy (*C*) and PVY infected (*D*) control grafts (C/C), healthy (*E*) and PVY infected (*F*) transgenic rooted plants (T), and healthy (*G*) and PVY infected (*H*) transgenic grafts (T/C) in the late stage of the infection (14 days). The scale bar indicates the NPQ intensity of the leaf pixels from high (white) to low (black) values.

conditions (Strasser *et al*. 2004). In our study, $PI_{(abs)}$ proved to be much more sensitive parameter of the influence of the PVY infection than commonly used maximum quantum yield of primary photochemistry (φ_{P0} and/or F_v/F_m). Only the insignificant decrease of φ_{P0} was found in all plant types after 14 DAI with the values that did not indicate the presence of any severe stress or impairment of PSII. This is in accordance for example with observation of Guo *et al.* (2005), who found no changes in φ_{P0} in *Turnip mosaic virus* infected stem of mustard plants despite a reduction in photosynthesis and growth. $PI_{(abs)}$ takes, apart from F_0 and F_m, also V_J and M_0 into account and it is thus dependent on the shape of the fluorescence transient. Higher sensitivity of $PI_{(abs)}$ as an indicator of plant stress was also found in several studies applying abiotic stress (*e.g*. drought stress – Živčák *et al.* 2008, cadmium stress – Van Belleghem 2007).

The infection with some strains of *Tobacco mosaic virus* is known to lead to a synthesis of viral coat protein inside chloroplasts and to its inhibitory association with PSII (Hodgson *et al*. 1989, Reinero and Beachy, 1989). As we did not prove the presence of PVY inside the chloroplasts (Schnablová *et al*. 2005), the negative effects on PSII might be due to lowered efficiency of protein repairing mechanisms (mainly for turnover of D1 protein) due to extensive synthesis of viral proteins or an impaired transport of nuclear-encoded chloroplast proteins into chloroplasts.

Nonphotochemical quenching (Chl-FI): The progression of PVY infection was followed by capturing images of different Chl-FI parameters from leaves of the healthy and PVY infected plants. Among other calculated parameters, NPQ was found to maximize the differences between the healthy and infected plants. Images NPQ5 (the third pulse of saturating light 5 s after the onset of actinic illumination), NPQ10 (the pulse 4 after 10 s), NPQ20 (the pulse 5 after 20 s), NPQ60 (the pulse 7 after 60 s), and NPQ300 (the pulse 12 after 300 s) were chosen as they sufficiently represented the NPQ kinetics as well as contrast between the healthy and PVY infected plants.

NPQ kinetics during the fluorescence transient measured in leaves of the healthy C, C/C, T, and T/C plants, respectively, is shown in Fig. 5*A,C,E,G*. The healthy C and C/C showed rather homogenous NPQ pattern over the leaf surface. NPQ values of the healthy C increased steeply up to a local maximum at 30 s and then after a slight decline at 60 s gradually increased up to 300 s. The healthy C/C reached the first maximum already at 20 s and after a decline at 30 s continued without any major changes up to 300 s. Both transgenic plant types (T, T/C) exhibited higher heterogeneity in NPQ pattern over the measured leaf area and much lower NPQ values in the early phase of the NPQ transient than control plant types (C, C/C). NPQ in the healthy T continued to rise up to 60 s, then declined to a local minimum at 180 s and then rose again slowly to 300 s. Unlike T plants, there was a steep increase in NPQ in T/C already at 10 s and the values rose up to the maximum at 60 s and then declined slowly up to 300 s. Starting at 20 s, T/C plants reached the highest NPQ values from all examined plant types.

In the early stage of the infection (3 DAI), no difference in NPQ images was found between the healthy and infected plants in any examined plant type (data not shown). In the middle stage of the infection (7 DAI), the first changes in NPQ pattern were observed in C and C/C, but clearly on the leaves where eye visible symptoms already started to develop (data not shown). The fact that no changes of Chl-FI parameters were observed before the formation of the first visible symptoms held Chl-FI

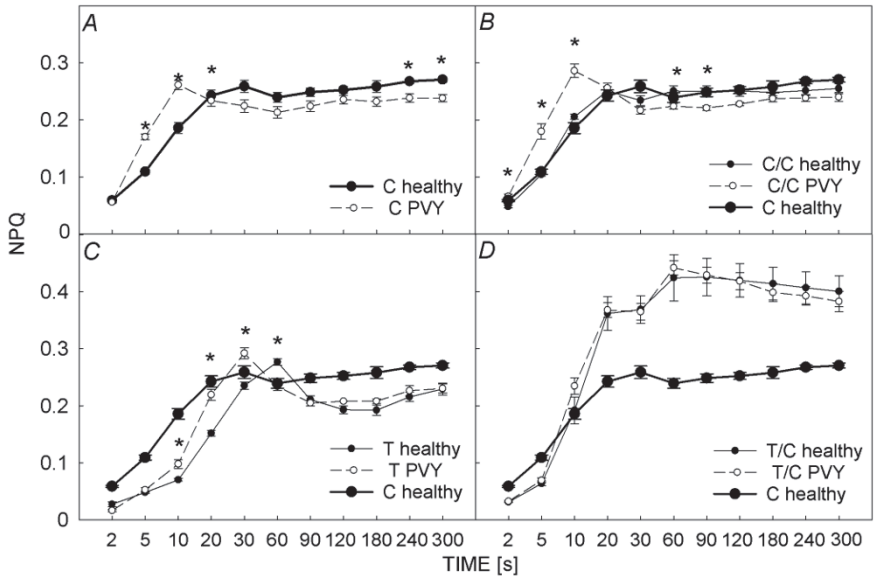

Fig. 6. Nonphotochemical quenching time-course measured in 10 × 10 pixel region of interest (ROI) from healthy and PVY infected control rooted plants – C (*A*), control grafts – C/C (*B*), transgenic rooted plants – T (*C*), and transgenic grafts – T/C (*D*) in the late stage of the infection. The values are means ± SE. Statistically significant differences between healthy and PVY infected plants are marked by an asterisk (P<0.05).

inapplicable in a presymptomatic detection of PVY infection in tobacco plants. Neither visible symptoms nor changes in NPQ pattern were observed in T and T/C in the middle stage of the infection. In the late stage of the infection (14 DAI), all C and C/C plants showed symptoms of the infection, T plants were less affected, and T/C plants showed no visible symptoms. Fig. 5 (*B,D,F,H*) displays the most informative images of the NPQ kinetics in leaves of the infected C, C/C, T, and T/C plants on 14 DAI. The infected C and C/C plants showed a very similar time varying, spatial pattern of NPQ processes. NPQ5 images already revealed areas of higher NPQ values surrounding the leaf veins with the maximum intensity at 20 s. Higher NPQ values were observed close to the leaf veins in infected T plants at 20 s but far from the extent found in both nontransgenic types. No differences in NPQ pattern were observed between the healthy and infected T/C plants. In addition, NPQ kinetics was followed in randomly chosen 10 × 10 pixel ROIs in the most contrasting areas adjacent to the leaf veins in order to reduce the influence of varying surface properties (Fig. 6). Acquired transients well corresponded with above-mentioned NPQ images. Concerning C and C/C plants, the most marked differences between the healthy and PVY infected plants were found at 5 and 10 s with significantly higher NPQ in the infected plants. In accordance with the images, no significant changes in NPQ were found between the healthy and infected T/C.

Generally, T/C plants showed significantly higher NPQ than other plant types. This corresponded with our data obtained from Chl *a* transient kinetics. Therefore, higher portion of an excitation energy absorbed in T/C, which was not used for further photochemical electron transport, must be dissipated by nonphotochemical processes, involved in NPQ.

As it is known that the virus spreads through the vascular tissue, we can assume that the areas with higher NPQ adjacent to the leaf veins corresponded to higher virus accumulation in the leaf tissue. It is supported by a correlation between NPQ amplification and a virus localization that was found by tissue-print in *Nicotiana benthamiana* plants infected with the Italian strain of the *Pepper mild mottle tobamovirus*, suggesting that an increase in the local NPQ values is associated with the areas invaded by the pathogen (Pérez-Bueno *et al*. 2006).

Conclusions: Our present experiments proved that transgenic tobacco (particularly T/C) was less affected by PVY infection, even if some significant changes in PSII functioning were induced most probably due to effects of the genetic transformation itself and/or due to the overproduction of CK. The most typical effects were namely the increase in the number of inactive PSII centres and a higher dissipation of energy *via* nonphotochemical processes. The significant negative effects of PVY on the photosynthesis of tobacco plants could be found only at the stage of the infection when visible symptoms already occurred. Chl fluorescence imaging method was not able to detect any presymptomatic changes in the infected tobacco; therefore this method cannot be used for such detection in this particular cultivar and virus interaction.

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