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Effects of biotic stress caused by *Potato virus Y* on photosynthesis in *ipt* transgenic and control *Nicotiana tabacum* L.

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Abstract

We studied the effect of biotic stress caused by *Potato virus Y^NTN* (PVY) on photosynthesis in transgenic P*ssu-ipt* tobacco overproducing endogenous cytokinins (CK) in comparison with control (non-transformed) plants. Both control and transgenic tobacco were grown as rooted or grafted plants. Content of viral protein increased significantly in control tobacco within ca. 18 days after inoculation, whereas transgenic plants exhibited much lower accumulation. This corresponded also with the presence of visible symptoms of PVY infection; while they were always present in control, rooted tobacco, they never developed in transgenic grafts. Contents of CKs (mostly in the forms of *N*- and/or *O*-glucosides) increased in all infected plants except transgenic grafts, where the highest amount of CKs was found already prior the inoculation. The photosynthetic rate (P_N) was significantly inhibited by PVY infection in control and transgenic rooted plants, while both grafted types were less affected. Reduction of P_N was caused not only by stomata closure, but also by the decrease of ribulose-1,5-bisphosphate carboxylase/oxygenase activity, contents of chlorophylls and xanthophyll cycle pigments, and activity of photosystem II (PSII). The negative effect on PS II was promoted by high irradiance treatment particularly in both rooted types infected by PVY.
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Keywords: *Potato virus Y*; Photosynthesis; Transgenic tobacco; *ipt*; Cytokinins

1. Introduction

Plant virus diseases have highly damaging effects on crop productivity [1]. *Potato virus Y* (PVY) belongs to genus *Potyvirus* (family Potyviridae), the largest group of plant viruses [2]. PVY is highly variable due to a wide range of host species. PVY^{NTN} isolates belong to PVY^{N} subgroup according to their reaction with characteristic necrotic symptoms on *Nicotiana tabacum*. The isolates of PVY have a capacity to infect tobacco systemically. In tobacco, PVY^{NTN} causes the veinal necrosis in the leaves, occasionally also a leaf distortion and stem necrosis. *Potyvirus* genom is formed by a single molecule of RNA. It is translated into one protein molecule. Single functional proteins are released from that large polypeptide by three different viral proteases. This results in several proteins with different function including a coat protein (CP). Potyviruses induce *in vivo* formation of cytoplasmic and nuclear inclusions in host cells containing aggregates of viral proteins. For PVY infection the inclusions called ‘‘pinwheels’’ and ‘‘bundle-like’’ structures are typical [2]. PVY induce also the formation of non-crystalline amorphous inclusions within the cytoplasm of infected cells.

Most research concerning plant viruses has been directed towards understanding the structure, genetics, transport and localization of viruses in plants. However, much less is known about the impact of virus infection on host plant physiology [3].

Many studies reported that viral infections lead to sugar accumulation and alter photosynthetic capacity [4,5]. The symptoms of virus infection seem to correlate with the presence of viral proteins inside the plastids. Deterioration of chloroplast ultrastructure, pigment composition and electron transport can

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be attributed to the damage caused mostly to photosystem II (PSII) during virus infection [6].

Virus infected plants display also a wide range of such symptoms that could be linked to the action, or reduction in action, of plant hormones [7]. As concern cytokinins (CK), Clarke et al. [8] found that 10 days after inoculation with *White clover mosaic potexvirus* total CK content was similar as in control plants. Nevertheless, 3 days following inoculation when virus content began to increase, the contents of CK free bases and ribosides declined. This could indicate that the decline in active CKs is needed prior to virus replication. Further studies proved that supplementing the xylem stream with low concentration of CKs inhibited virus replication at the dsRNA level [9,10], and also prevented virus-induced decline in several enzymes involved in the scavenging of free radicals [11].

Transgenic plants with the bacterial gene for isopentenyltransferase (*ipt*), a key enzyme of CK synthesis pathway, accumulate higher contents of endogenous CKs [12]. This affects plant growth, development, and senescence and also the resistance to abiotic and biotic stresses [13,14].

In present study, we have used P*ssu-ipt* tobacco to study the effects of *Potato virus Y* on photosynthetic and water relation characteristics of transgenic plants with elevated content of endogenous CKs. The main aim of this study was to find out, if high content of CKs could improve the resistance of transgenic plants against the PVY infection or diminish its negative impacts on photosynthesis. We correlated our findings with CK contents in both healthy and virus-infected plants.

2. Material and methods

2.1. Plant material

Control tobacco (*N. tabacum* L. cv. Petit Havana SR1) was grown as rooted plants (C) from seeds or as grafted onto control rootstock (C/C). Transgenic tobacco (P*ssu-ipt*) containing a supplementary *ipt*-gene under a control of the promoter for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) was generated by means of the *Agrobacterium tumefaciens* transformation system and grown *in vitro* as shoots unable to form roots. The transgenic shoots were grafted on C rootstock and grown as grafts (T/C) as described by Beinsberger et al. [15]. P*ssu-ipt* transgenic plants (T), i.e. the autogamic progeny of the transgenic grafts, which are able to form a small root system, were grown from seeds, selected on agar medium with kanamycin (*in vitro*) and then transferred into soil. Although the growth and development of transgenic plants was slower than that of controls, they went throughout all developmental stages as controls.

All plants were grown after *in vitro* precultivation in pots with soil substrate in a greenhouse under temperature of 25 °C day/18 °C night, and relative humidity 60%. Natural photosynthetic photon flux density (PPDF) mean ca. 500 μmol (quanta) m^{-2} s^{-1} was prolonged by the additional illumination (*AgroSon T* and HT9 lamps, ca. 200 μmol (quanta) m^{-2} s^{-1}) to 16 h.

For inoculation, plants at early vegetative stage with total number of 4–5 leaves (C, 7–8 weeks old; T, 10–12 weeks old; C/C shoots 3–4 weeks after grafting; T/C shoots 4–6 weeks after grafting) were used.

2.2. Inoculation of plants with PVY^{NTN} isolate

Mature leaves at the bottom of the plant were mechanically inoculated with virus isolate of PVY^{NTN} (Lebanon, provided by Dr. P. Dědič—Institute of Potato Research, Havlíčkův Brod, Czech Republic) on the adaxial surface. Leaf samples from infected plants were taken ca. 15–18 days after the inoculation from young symptomatic mature leaves. Samples from healthy plants were taken at the same time from the same leaf insertion level.

2.3. DAS-ELISA

Leaf samples were frozen in liquid N_2 and stored at −75 °C. The extent of viral infection was determined by DAS-ELISA [16] in homogenates of the leaves of infected and control plants using polyclonal antibodies raised against the PVY [17].

2.4. Cytokinin extraction and purification

CKs were extracted overnight at −20°C with Bieleski solvent [18] from leaves (1 g), grounded under liquid nitrogen. For MS quantification, deuterium-labelled cytokinins ([2H5]Z, [2H5]ZR, [2H5]Z-7G, [2H5]Z-9G, [2H5]Z-OG, [2H5]ZR-OG, [2H3]DZ, [2H3]DZR, [2H6]iP, [2H6]iPR, [2H6]iP-7G, [2H6]iP-9G; Apex, UK) were added as internal standards. After centrifugation, the extracts were purified using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA) and evaporated to water phase. After acidifying with HCOOH, CKs were trapped on an Oasis MCX mixed mode, cation exchange, reverse-phase column (150 mg, Waters Corporation, Milford, MA, USA) [19]. After two washes (with 1 M HCOOH and 100% MeOH), CK phosphates (CK nucleotides) were eluted with 0.17 M NH_4OH in water, further CK bases, ribosides, and glucosides were eluted with 0.17 M NH_4OH in 60% (v/v) MeOH. The latter eluate was evaporated to dryness. NH_4OH was evaporated from the eluted fraction with CK nucleotides. About 0.1 M Tris (pH 9.6) was added to samples and after treatment with alkaline phosphatase (30 min at 37 °C) CK nucleotides were analysed as their corresponding ribosides. After neutralization, the solution was passed through a C18 Sep-Pak cartridge. CKs were eluted with 5 ml 80% (w/w) methanol and evaporated to dryness. Samples were stored at −20 °C until further analysis.

2.5. Quantitative analysis of cytokinins

Purified CKs samples were analysed by LC–MS system consisting of HTS PAL autosampler (CTC Analytics, Switzerland), Rheos 2000 quaternary pump (FLUX, Switzerland) with Csi 6200 Series HPLC Oven (Cambridge Scientific Instruments, England) and LCQ Ion Trap mass spectrometer

(Finnigan, USA) equipped with an electrospray. About 10 μl of sample were injected onto a C18 column (AQUA, 2 mm × 250 mm × 5 μm, Phenomenex, USA) and eluted with 0.0005% acetic acid (A) and acetonitrile (B). The gradient profile was 5 min 10% B, then to 17% in 10 min, then to 46% in 10 min at a flow rate of 0.2 ml/min. Column temperature was kept at 30 °C. The effluent was introduced in mass spectrometer being operated in the positive ion, full-scan MS/MS mode. Quantification was performed using a multilevel calibration graph with deuterated CKs as internal standards.

2.6. Scanning electron microscopy (SEM)

Leaves of healthy and infected plants were collected ca. 6 h after the onset of irradiation period. They were cut into small pieces and infiltrated for 1 h in fixation medium (0.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2). Samples were transferred to 50% acetone and then dehydrated in ascending acetone series. Samples were dried by CPD method (Pelco CPD2), coated by gold and examined in scannig electron microscope Jeol 6300 (Jeol, Japan) with TESCAN System for image analysis.

2.7. Photosynthetic parameters

Chlorophyll *a* fluorescence kinetics was measured on the adaxial surface of attached leaves after a 25-min dark period with the *PAM Chlorophyll Fluorometer* (*Walz*, Effeltrich, Germany) at room temperature and ambient CO_2 concentration. Measuring beam PPFD was 0.35 $\mu mol\ m^{-2}\ s^{-1}$, actinic irradiance 200 $\mu mol\ m^{-2}\ s^{-1}$, and 700-ms saturated flashes of "white light" (2500 $\mu mol\ m^{-2}\ s^{-1}$) were applied at 300 s intervals. Data sampling, control and calculation were served by the *DA 100 Data Acquisition System* (*Walz*, Effeltrich, Germany) (for detail, see Ref. [20]). The nomenclature of van Kooten and Snel [21] and Osmond et al. [22] was used throughout the work.

Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s) were measured using the gas exchange system LCA-4 (ADC *Bio Scientific*, Hodderdon, England) at a temperature of 25 °C, irradiance of 750 $\mu mol\ m^{-2}\ s^{-1}$, relative humidity of 50% and CO_2 concentration of 350 $\mu mol\ mol^{-1}$.

Relative water content (RWC) was measured gravimetrically using leaf discs (0.5 cm^2) water-saturated in moistened polyurethane foam in darkness [23].

Chlorophyll *a* fluorescence, gasometric parameters, and RWC were measured ca. 15–18 days after inoculation by PVY and the first mature symptomatic leaf attached to the plant was used for all measurements.

2.8. Pigment determination

Contents of photosynthetic pigments were determined in acetone extracts of leaf discs (3.8 cm^2) by HPLC (Spektra-Physics, San Jose, USA) using a reverse phase column (Sepharon SGX C18, Tessek, CR). The solvent system was acetonitrile:methanol:water (80:12:6) followed by 100% methanol, and the gradient was run from 8 to 12 min. The flow rate was 1 $mm^3\ s^{-1}$, the detection wavelenght was 445 nm.

2.9. High irradiance experiments (HL)

Healthy and PVY infected plants ca. 15–18 days after the inoculation were transferred from the greenhouse conditions to the chamber, where they were exposed to irradiance of 1000 μmol (quanta) $m^{-2}\ s^{-1}$ for 1 h at 25 °C. After that treatment chlorophyll *a* fluorescence kinetics was measured on attached leaves as mentioned above and samples for pigment determination were taken from plants. Then all plants were placed into the greenhouse conditions and after one more hour, chlorophyll *a* fluorescence kinetics was measured again.

2.10. Activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO)

The activity of RuBPCO was measured in crude extracts obtained from leaves after homogenization in medium containing 1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) in 100 mM Tris–HCl (pH 7.8) and centrifugation at 18,000 × *g* for 15 min at 4 °C. After 10 min preincubation of extracts in 100 mM Tris–HCl pH 8.1, 1 mM EDTA, 30 mM $MgCl_2$, 5 mM DTT, and 5 mM $NaHCO_3$ at 23 °C, 1 mM RuBP and 1 mM $Na_2{}^{14}CO_3$ were added. The reaction was stopped after 5 min by adding 0.5 cm^3 6M HCl. The RuBPCO activity was measured as ^{14}C fixed into the acid stable reaction product in a toluene scintillation coctail on a Beckman LS 6000 SE liquid scintillation counter. The activity was measured at 23 °C and calculated as mU (1 mU catalyses production of 1 nmol products per min) per gram of fresh leaf matter according to [24].

Soluble protein content was determined according to Ref. [25].

2.11. Statistical analysis

Leaves for the photosynthetic activity, RuBPCO, pigment, and chlorophyll *a* fluorescence determination were taken from at least five plants of each plant type cultivated and inoculated in four independent series. SEM was carried out on the leaf samples from two independent series. LC–MS analysis of CKs was done in the leaf samples from three independent series. Statistically significant differences in the mean values were tested by ANOVA or Student's *t*-test at $P = 0.05$.

3. Results

3.1. Virus infection

The virus content was determined by DAS-ELISA. Absorbance at 450 nm corresponding to relative virus content increased significantly in both control types of infected plants (1.85 ± 0.1 in C; 1.5 ± 0.4 in C/C) within 15–18 days following the inoculation. In transgenic rooted plants (T),

virus accumulated less than in controls (0.83 ± 0.2), nevertheless, it was higher than in T/C (0.220 ± 0.01). The development of symptoms of viral infection, i.e., veinal and stem necrosis and chlorosis, correlated with the accumulation of virus in plant cells. The symptoms were always visible in C, whereas in T and C/C it was more dependent on plant age at the time of inoculation, i.e. the younger plants often expressed the symptoms, whereas they were less frequent in the older plants. No symptoms of infection were observed in T/C.

3.2. Cytokinin contents

Changes in CK contents were examined ca. 18 days after the inoculation by PVY (Table 1). Besides total CKs, we compared also the changes in contents of *N*- and *O*-glucosides. In C, the total CK content increased in infected plants (233%) with slightly increasing portion of *N*-glucosides (from 55% of total CKs in healthy tobacco to 62% in infected plants). No significant changes in amounts of CK free bases and ribosides were found in PVY infected C. In C/C, the total content of CKs was moderately higher in healthy plants compared with C and the enhancement after PVY infection was similar (ca. 213%). However, in infected C/C the portion of *N*-glucosides increased from 11 to 25% and *O*-glucosides from 28 to 63% of total CKs. The content of CK free bases and ribosides significantly decreased in C/C after PVY infection. In both transgenic types, total amount of CKs was at least 10 times higher in healthy plants compared with controls. In T/C, the enhancement even exceeded the upper detection limits of LC–MS method, therefore the values should be cautiously evaluated. Therefore, contrary to other plant types, no significant change in total CKs was detected in T/C after PVY infection, although the increase of contents of free bases and ribosides was observed in the infected T/C. In T, the total content of CKs doubled after the infection and the portion of *N*-glucosides increased to 66% compared with 38% of the total amount in healthy T. The portion of CK free bases and ribosides slightly decreased after PVY infection.

Table 1
The content of endogenous CKs (pmol g^{-1} FM) in healthy control rooted plants (C), control grafts (C/C), P*ssu-ipt* transgenic rooted tobacco (T), and transgenic grafts (T/C) and PVY infected by ca. 18 days after the inoculation

Plant	Total CKs	*N*-Glucosides	*O*-Glucosides	FB + RB
C	36.0 a	19.8 a	9.2 a	2.8 a
C-PVY	83.9 ab	52.4 b	22.4 a	3.9 a
C/C	87.0 ab	10.1 a	24.2 a	42.5 b
C/C-PVY	185.2 b	47.0 ab	116.5 b	10.1 a
T	478.9 c	183.7 c	37.5 a	123.9 c
T-PVY	801.2 d	529.1 d	124.1 b	99.5 bc
T/C	8920 e	4781 e	3422 c	369.4 d
T/C-PVY	7885 e	4321 e	1490 c	1704 e

The values represent the means of three replicates. The S.D. values averaged 8% and did not exceed 17% of the mean. Total CKs represent the sum of all CKs; *N*-glucosides = *trans*-zeatin-7-glucoside, *trans*-zeatin-9-glucoside, dihydrozeatin-7-glucoside, dihydrozeatin-9-glucoside, isopentenyladenine-7-glucoside, isopentenyladenine-9-glucoside; *O*-glucosides = *trans*-zeatin-9-riboside-*O*-glucoside, *trans*-zeatin-*O*-glucoside, dihydrozeatin-9-riboside-*O*-glucoside; FB + RB = free bases and ribosides = *trans*-zeatin, *cis*-zeatin-9-riboside, isopentenyladenine, isopentenyladenine-9-riboside; ribotides and dihydrozeatin are included in the total sum of CKs.

3.3. Stomata

SEM examination did not show any significant differences in a shape or a size of stomata between control and transgenic plants (Fig. 1). However, the stomata of healthy controls were mostly open during daytime (Fig. 1A and B), whereas those in transgenic plants were mostly closed (Fig. 1E and F). PVY infection caused that stomata of all infected plants were predominantly closed (Fig. 1C, D, G and H).

3.4. Gas exchange

Gas exchange parameters showed significant differences between control and transgenic tobacco (Fig. 2). Healthy T and T/C exhibited similarly ca. 50% lower rates of net photosynthesis (P_N) compared with C. Reduction of P_N was found also in healthy C/C (Fig. 2A). In PVY-infected C, P_N declined to 15% of healthy controls, while the decrease of P_N caused by PVY infection was ca. 50% in transgenic plants. Transpiration rates (E) were lower in healthy C/C, T, and T/C, but decreased less after the PVY infection compared with C (Fig. 2B). Stomatal conductance (g_s) was the highest in healthy C and decreased more significantly after PVY infection than in other plant types (Fig. 2C). Relative water content (RWC) decreased significantly in both control types infected by PVY, while the infection had no effect in T and T/C (Fig. 2D).

3.5. Chlorophyll *a* fluorescence kinetics and quenching analysis

Generally, chlorophyll *a* fluorescence parameters were significantly affected by PVY only in both rooted plant types. In both grafted types, the changes were mostly statistically insignificant (Figs. 3 and 4). Quantum yield (Φ_{II}) decreased significantly in both rooted types infected by PVY, while in both grafted types did not change (Fig. 3A). Non-photochemical quenching (q_N) was moderately reduced in all infected plants (Fig. 3B), but the statistically significant decrease in q_N was found only in C. The significant increase in the reduction status of Q_A corresponding to the number of closed reaction PSII centres ($1 - q_P$) was observed also in PVY infected C and T (Fig. 3C). Nevertheless, T/C exhibited the highest $1 - q_P$ independently on the viral infection. Vitality index (Rfd) was lower in healthy grafted and both transgenic types compared with C (Fig. 3D). The significant reduction of Rfd was observed in C and T after PVY infection, while in both grafted types (C/C and T/C) the changes were only marginal.

Only moderate decline was found in maximal photochemical efficiency of PSII (F_v/F_M) in infected C/C and T/C (Fig. 4). In C, F_v/F_M decreased to ca. 77% of healthy control, whereas in transgenic T the decline was less significant (Fig. 4).

Healthy and PVY infected plants exposed to high irradiance (HL) for 1 h showed photoinhibition that caused the significant decline in F_v/F_M (Fig. 4), the moderate increase in q_N, and in

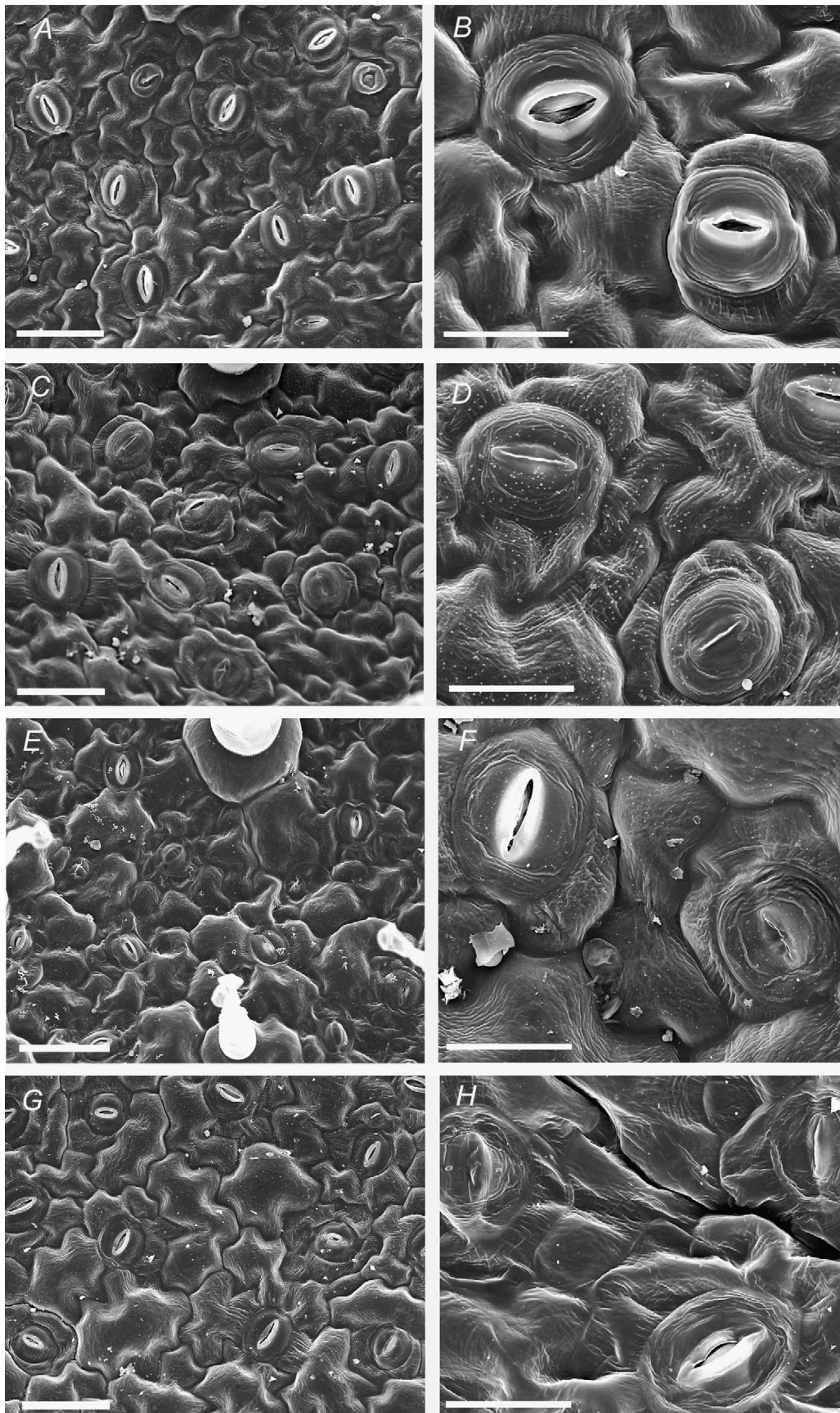

Fig. 1. Stomata from adaxial side of tobacco leaves examined by scanning electron microscope. The structure of stomata from healthy control rooted plants (A and B), PVY infected control plants (C and D), healthy transgenic rooted plants (E and F), and PVY infected transgenic plants (G and H). Scale bars = 50 μm (A, C, E and G) and 20 μm (B, D, F and H).

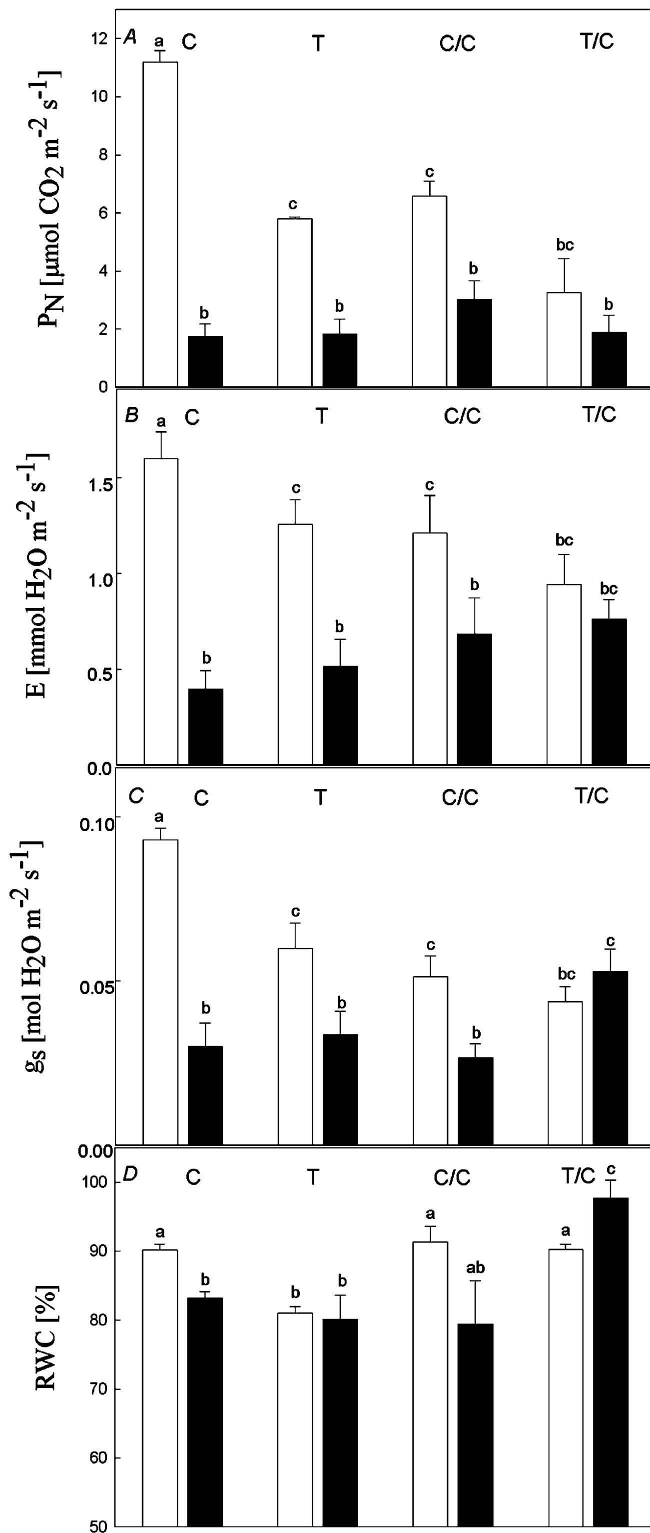

Fig. 2. Photosynthetic parameters of healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). (A) Net photosynthetic rate (P_N); (B) transpiration rate (E); (C) stomatal conductances (g_s); (D) relative water content (RWC). The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

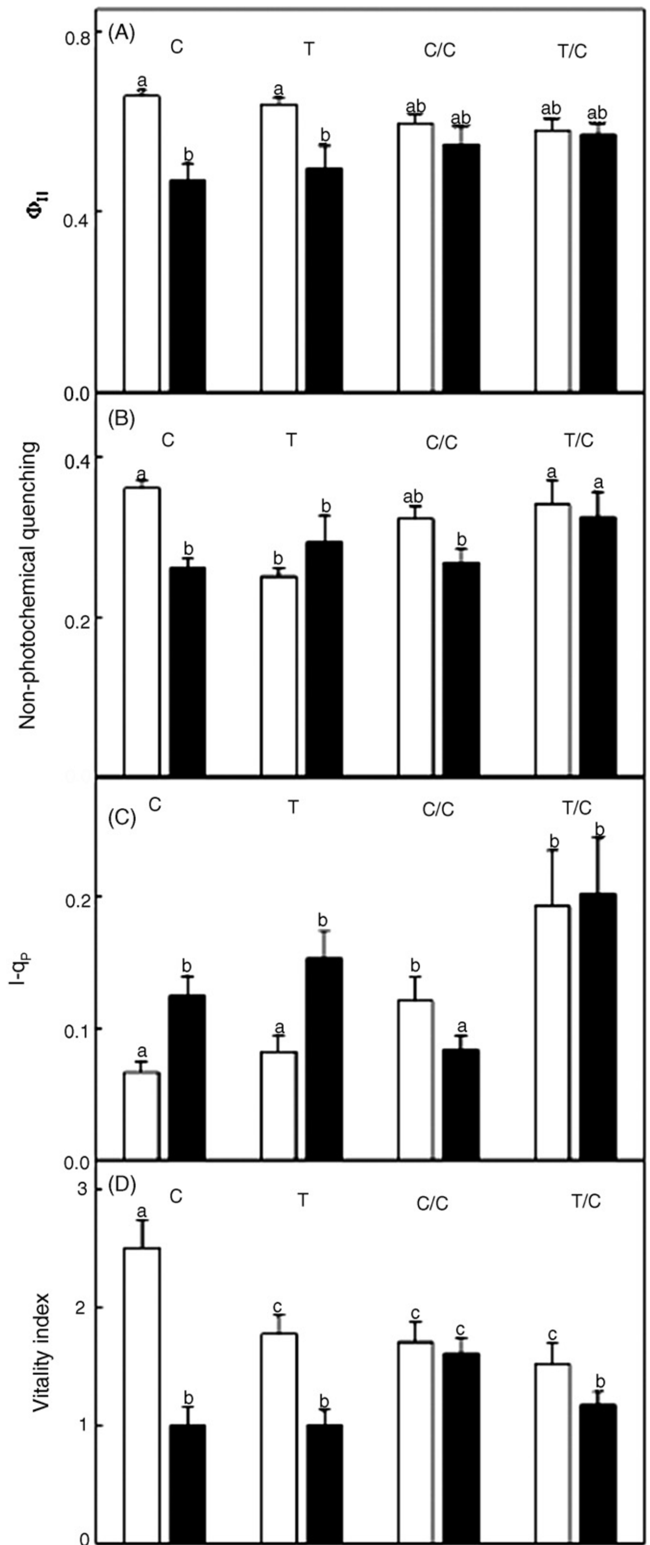

Fig. 3. Parameters of chlorophyll *a* fluorescence kinetics in healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). (A) Quantum yield (Φ_{II}); (B) non-photochemical quenching (q_N); (C) the portion of closed reaction centers ($1 - q_P$); (D) Rfd, vitality index. The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

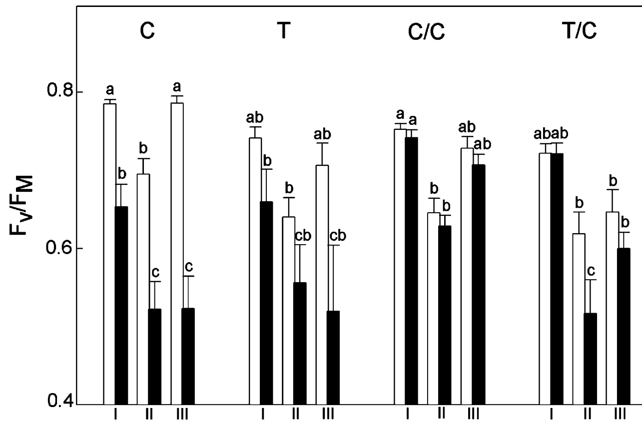

Fig. 4. Maximal photochemical efficiency of PS II (F_v/F_M) in healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). I, plants from greenhouse conditions; II, plants after 1 h of HL (ca. 1000 μmol m^{-2} s^{-1}); III, plants after 1 h in greenhouse condition after HL. The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

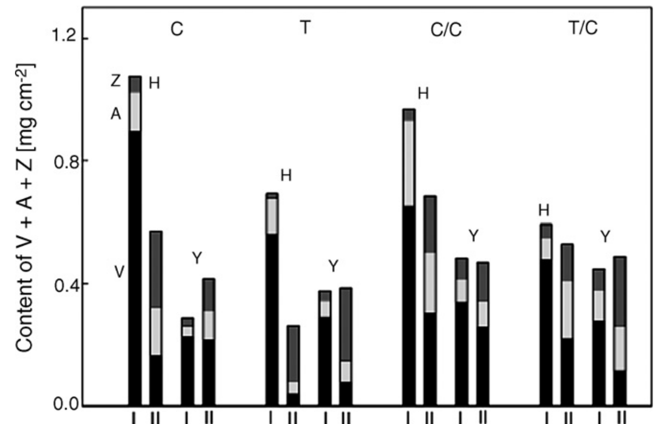

Fig. 6. Changes in xanthophyll cycle pigments in healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). A, anteraxanthin; V, violaxanthin; Z, zeaxanthin. I, plants from greenhouse conditions; II, plants after 1 h of HL (ca. 1000 μmol m^{-2} s^{-1}). The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

$1 - q_P$ (not shown). In healthy plants, chlorophyll *a* fluorescence parameters reached after 1 h relaxation under the greenhouse condition almost the same values as prior HL. However, in PVY infected C, T, and TC plants photoinhibition effect of HL persisted after 1 h relaxation. This was less significant in both grafted types (Fig. 4).

3.6. Pigment analysis

The decline of chlorophyll content was another symptoms of PVY infection. Only T/C maintained unchanged chlorophyll $a + b$ content (Fig. 5). It was already slightly lower prior the infection compared with other plant types. In all other plant types chlorophyll decreased significantly after PVY infection to ca. 60% found in healthy plants.

The content of xanthophylls, i.e., violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z), was also significantly reduced in all PVY infected plants except T/C, where the decline was only marginal (Fig. 6). The portion of individual xanthophylls changed moderately by PVY infection. The only exception was T, where A content significantly decreased compared with healthy plants.

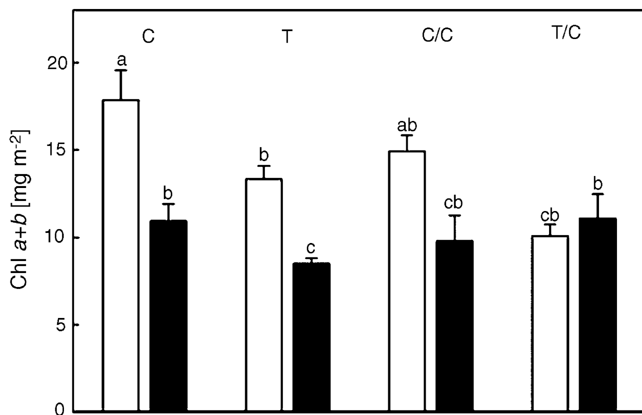

Fig. 5. Total chlorophyll content in healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

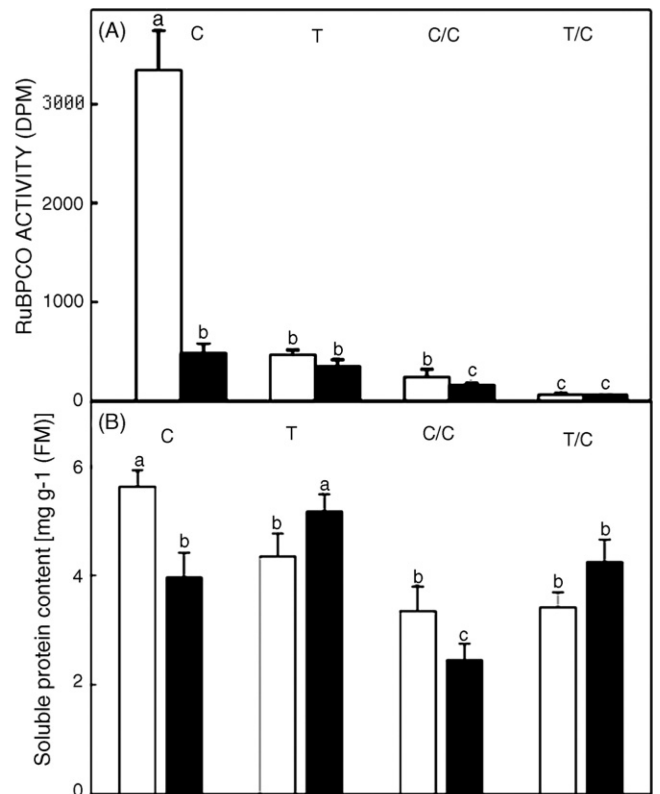

Fig. 7. Changes in activity of RuBPCO (A) and in soluble protein content (B) in healthy (open columns) and PVY infected (closed columns) control, rooted tobacco (C), transgenic rooted plants (T), control grafts (C/C), and transgenic grafts (T/C). The values are means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

HL caused the accumulation of Z on the expense of V in all HL exposed plants. The most significant effect was found in T, where the highest accumulation of Z was observed. In PVY infected plants, HL treatment did not change the total amount of xanthophylls contrary to healthy plants, where HL usually caused the reduction in their total amount. T/C plants exhibited only insignificant changes in xanthophylls compared with all other plant types.

3.7. RuBPCO activity

Healthy C plants exhibited the highest RuBPCO activity compared with other plant types (Fig. 7A). T and C/C plants showed more than 70% lower activity than C. Even lower activity was detected in samples from T/C. PVY infection caused the most significant decline in C (ca. 20% of healthy control), while in other types no change (T/C) or 10–15% (C/C and T) reduction of activity compared with healthy plants was observed.

Soluble protein content of leaf extracts decreased significantly in both control types (C and C/C) after PVY infection, while in both transgenic types moderate increase was found in the infected plants (Fig. 7B).

4. Discussion

Our experiments proved that transgenic P*ssu-ipt* tobacco was less susceptible to PVY infection than control plants. Moreover, grafted plants, both control and transgenic, were also less sensitive to the infection. This can be deduced not only from the occurrence of the infection symptoms, but also from less affected physiological and biochemical parameters of infected plants. However, in both transgenic (i.e. T, T/C) and both grafted types (i.e. C/C, T/C), some reduction in photosynthesis was found prior PVY infection.

The symptom development highly corresponded to virus protein content that was found in infected plants. Very low amount of viral proteins accumulated in T/C, where no symptoms were observed. The part of T and C/C plants, particularly those inoculated slightly later during the plant ontogeny, did not also exhibit any or very mild symptoms of infection. This was shown in previous paper [26], where the data obtained from those non-symptomatic plants were included. Those infected plants without symptoms of PVY infection showed usually also lower amount of viral proteins.

CK contents increased in all infected plants but T/C, where CK content was excessive prior the inoculation and no further changes were detected. Nevertheless, this confirms our previous findings that in T/C, CK content was much higher than in transgenic rooted plants in this stage of the plant development [27]. Generally, the increase in total CK contents in PVY infected plants was mostly due to the increase in inactive (*N*-glucosides) or storage forms (*O*-glucosides) of CKs. In C, the proportional increase in both *N*- and *O*-glucosides was observed. Small or no changes were found in CK isoprenoid free bases and ribosides, which are accounted for active forms of CKs. In C/C, the increase in total CKs correlated with the enhancement of *O*-glucosides, while in T, a higher portion of *N*-glucosides caused the increase in total CK content after PVY infection. Nevertheless, in both C/C and T the portion of active CKs decreased. This corresponds with results of Clarke et al. [8], who proposed that a reduction in these CK forms may account for some of the symptoms exhibited by virus-infected plants and may also play a role in virus replication. However, it seems that higher CK level prior the virus attack could help alleviate the infection impact and retard the virus replication and the symptom development [28]. In T/C, where the highest contents of CKs and even the increase in the portion of active CKs were found, no symptoms of PVY infection were detected. In T, in a developmental stage, when plants were inoculated, the CK level was not probably high enough to fulfill completely this role, whereas in T/C, mechanisms involved usually in CK homeostasis are partially inhibited and thus the CK concentration exceeds the critical level needed for the retardation of virus growth (see Table 1). CKs have been implicated as components of the plant defense signal transduction pathway with respect to wounding and virus infection [29]. Sano et al. [30] found that CKs interferred with the signal transduction mechanism participating in pathogenesis related (PR) protein synthesis by controlling endogenous levels of salicylic and jasmonic acids. In P*ssu-ipt* tobacco, both PR-proteins and higher contents of salicylic acid, were already detected in healthy plants [31].

Photosynthesis of C plants was strongly inhibited by PVY infection. We proved that P_N was partially limited by stomata closure that occurred as a result of the infection. This has been previously shown in two tobacco cultivars infected by two different potyviruses, i.e. PVY and *Potato virus A* [32]. The negative effects of viral or bacterial pathogens on the rate of photosynthesis was reported also by many other authors (e.g. [33–35]). Arias et al. [33] found a decrease in photosynthetic rate, but no change in stomatal conductance in sunflower infected by *Sunflower chlorotic mottle virus*. In our experiments, RWC in C and C/C decreased after PVY infection (Fig. 2D). This might contribute to lowering g_s in those plants. The opposite effect of PVY infection was observed in T/C, where slightly higher RWC and g_s were found after PVY infection. Our previous experiments, where transmission electron microscopy was used for leaf sample examination, indicated also a high accumulation of viral aggregates inside the guard cells of infected controls [26]. This might negatively affect the stomata functioning contrary to both transgenic types, where was lower accumulation of viral proteins. In addition, pathogen induced production of H_2O_2, which was also proved in our experiments (Synková, unpublished), cannot be excluded and this might contribute to stomatal closure [36,37]. As a consequence of decreased g_s, E, and P_N decreased in all PVY infected plants, but percentage of P_N decline was much higher than that of E. Therefore water use efficiency (WUE = P_N/E) decreased considerably after PVY infection.

Moreover, not only stomatal limitations took part in the inhibition of photosynthesis by PVY. Markedly lower carboxylating activity of RuBPCO, found in PVY infected plants during our experiment, has been observed in plants under

various stresses [38] and also after pathogen attack [35]. This could be partly due to a reduction in intracellular leaf CO_2 concentration caused by partially closed stomata and decreased diffusion of CO_2 to chloroplasts from substomatal cavities. However, in control PVY infected plants, soluble protein content also decreased. This could indicate also the loss of RuBPCO proteins which comprises ca. 50% of soluble proteins in leaves.

In PVY infected plants, the function of PSII was also negatively influenced, which was demonstrated by the decline in both maximal photochemical efficiency (F_v/F_M) and quantum yield (Φ_{II}), and a higher number of closed PSII centres, namely in PVY infected C plants. This was also observed in *Nicotiana benthamiana* infected by pepper and paprika mild mottle viruses [39]. In our experiment, this effect was particularly pronounced by HL treatment, when PVY infected plants exhibited limited or slower ability to recover. The association of the viral coat protein or some other products synthesized as a result of the infection with PSII complex or oxygen-evolving complex have been suggested as a reason for decline in PSII electron transport [6]. However, we did not prove the presence of PVY inside the chloroplasts [26]. Therefore the reason for negative effects on PSII might be due to lowering the efficiency of protein repairing mechanisms (i.e. namely for turnover of D1 protein) due to extensive synthesis of viral proteins that could be synthesized preferentially and/or an impaired transport of nuclear-encoded chloroplast protein into chloroplasts. The significant role in deterioration of photosynthetic apparatus can also play the decrease in total chlorophyll and xanthophyll contents in PVY infected plants. Decline in chlorophyll content seems to affect directly chloroplast functioning in, e.g. *Tobacco mosaic virus* infected tobacco [40]. Conversions of xanthophylls cycle pigments are involved in the photoprotective dissipation mechanisms under HL [41]. The decline in total xanthophyll contents due to PVY infection could explain higher sensitivity of infected plants to HL, although the content of zeaxanthin increased after HL treatment in both healthy and PVY infected plants.

Our results confirmed that high amount of endogenous cytokinins prior the inoculation could restrict the accumulation of viral proteins in infected plants and thus alleviate the damaging effects and development of infection symptoms. Nevertheless, overproduction of endogenous CKs resulted in a certain decline in photosynthesis prior PVY inoculation. Also grafting procedure prior the inoculation caused a stress response resulting also in negative affected photosynthesis.

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References

[1] G.N. Agrios, Plant Pathology, 3rd ed., Academic Press, New York, 1997 .

[2] D. Shukla, C.W. Ward, A.A. Brunt, The Potyviridae, CAB International, Wallingford, 1994, pp. 32–51.

[3] Z. Havelda, A. Maule, Complex spatial responses to Cucumber mosaic virus infection in susceptible *Cucurbita pepo* cotyledons, Plant Cell 12 (2000) 1975–1985.

[4] S. Balachandran, V.M. Hurry, S.E. Kelley, C.B. Osmond, S.A. Robinson, J. Rohozinski, G.G.R. Seaton, D.A. Sims, Concepts of plant biotic stress. Some insights into stress physiology of virus-infected plants, from the perspective of photosynthesis, Physiol. Plant 100 (1997) 203–213.

[5] M. Šindelářová, L. Šindelář, L. Burketová, Changes in glucose, fructose and saccharose metabolism in tobacco plants infected with potato Y, Photosynthetica 42 (1999) 431–439.

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

[7] P. Jameson, Cytokinins and auxins in plant-pathogen interactions—an overview, Plant Growth Regul. 32 (2000) 369–380.

[8] S.F. Clarke, M.J. McKenzie, D.J. Burritt, P.L. Guy, P.E. Jameson, Influence of white clover mosaic potexvirus infection on the endogenous cytokinin content of bean, Plant Physiol. 120 (1999) 547–552.

[9] S.F. Clarke, D.J. Burrit, P.E. Jameson, P.L. Guy, Influence of plant hormones on virus replication and pathogenesis-related proteins in *Phaseolus vulgaris* L. infected with white clover mosaic potexvirus, Physiol. Mol. Plant Pathol. 53 (1998) 195–207.

[10] S.F. Clarke, D.J. Burrit, P.E. Jameson, P.L. Guy, Influence of plant hormones on white clover mosaic potexvirus double stranded RNA, Plant Pathol. 49 (2000) 428–434.

[11] S.F. Clarke, Physiological and molecular interactions between plant hormones and white clover mosaic virus infection of *Phaseolus vulgaris* L., PhD Thesis, Dunedin, University of Otago, New Zealand, 1996.

[12] H. Synková, N. Wilhelmová, Z. Šesták, J. Pospíšilová, Photosynthesis in transgenic plants with elevated cytokinin content, in: M. Pessarakli (Ed.), Handbook of Photosynthesis, Marcel Dekker, New York, 1997, pp. 541–552.

[13] H. Synková, K. Van Loven, J. Pospíšilová, R. Valcke, Photosynthesis of transgenic P*ssu-ipt* tobacco, J. Plant Physiol. 155 (1999) 173–182.

[14] H. Synková, R. Valcke, Response to mild water stress in transgenic P*ssu-ipt* tobacco, Physiol. Plant 112 (2001) 513–523.

[15] S. Beinsberger, R. Valcke, H.M. Clijsters, J.A. De Greef, H.A. Van Onckelen, Effects of enhanced cytokinin levels in *ipt* transgenic tobacco, in: M. Kamínek, D.W.S. Mok, E. Zažímalová (Eds.), Physiology and Biochemistry of Cytokinins in Plants, Academic Publishing, The Hague, The Netherlands, 1992, pp. 77–82.

[16] M.F. Clark, A.N. Adams, Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses, J. Gen. Virol. 34 (1977) 475–483.

[17] N. Čeřovská, Production of monoclonal antibodies to *Potato virus Y*^{NTN} strain and their use for strain differentiation, Plant Pathol. 47 (1998) 505–509.

[18] R.L. Bieleski, The problem of halting enzyme action when extracting plant tissues, Anal. Biochem. 9 (1964) 431–442.

[19] P.I. Dobrev, M. Kamínek, Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction, J. Chromatogr. 950 (2002) 21–29.

[20] J. Pospíšilová, N. Wilhelmová, H. Synková, J. Čatský, D. Krebs, I. Tichá, B. Hanáčková, J. Snopek, Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid, J. Exp. Bot. 49 (1998) 863–869.

[21] O. van Kooten, J.F.H. Snel, The use of chlorophyll fluorescence nomenclature in plant stress physiology, Photosynth. Res. 25 (1990) 147–150.

[22] C.B. Osmond, J. Ramus, G. Levavasseur, L.A. Franclin, W.J. Henley, Fluorescence quenching during photosynthesis and photoinhibition of *Ulva rotundata* Blid, Planta 190 (1993) 97–106.

[23] J. Čatský, Determination of water deficit in discs cut out from leaf blades, Biol. Plant 2 (1960) 76–78.
[24] M. Stiborová, S. Kšinská, A. Březinová, Effect of NaCl on the growth and biochemical characteristics of photosynthesis of barley and maize, Photosynthetica 21 (1987) 320–328.
[25] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[26] R. Schnablová, H. Synková, N. Čeřovská, The influence of Potato virus Y infection on the ultrastructure of Pssu-ipt transgenic tobacco, Int. J. Plant Sci. 166 (2005) 713–721.
[27] H. Synková, R. Pechová, R. Valcke, Changes in chloroplast ultrastructure in P*ssu-ipt* tobacco during plant ontogeny, Photosynthetica 41 (2003) 117–126.
[28] M. Dermastia, M. Ravnikar, Altered cytokinin pattern and enhanced tolerance to potato virus YNTN in the susceptible potato cultivar (*Solanum tuberosum* cv. Igor) grown in vitro, Physiol. Mol. Plant Pathol. 48 (1995) 65–71.
[29] H. Sano, Y. Ohashi, Involvement of small GTP-binding proteins in defense signal-transduction pathways of higher plants, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4138–4144.
[30] H. Sano, S. Seo, A. Koizumi, T. Niki, H. Iwamura, Y. Ohashi, Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants, Plant Cell Physiol. 37 (1996) 762–769.
[31] H. Synková, Š. Semorádová, L. Burketová, High content of endogenous cytokinins stimulates activity of enzymes and proteins involved in stress response in *Nicotiana tabacum*, Plant Cell Tissue Organ Cult. 79 (2004) 169–179.
[32] H. Ryšlavá, K. Muller, Š. Semorádová, H. Synková, N. Čeřovská, Photosynthesis and acktivity of phosphoenolpyruvate carboxylase in *Nicotiana tabacum* L. leaves infected by *Potato virus A* and *Potato virus Y*, Photosynthetica 41 (2003) 357–363.
[33] M.C. Arias, S. Lenardon, E. Taleisnik, Carbon metabolism in sunflower plants infected with the *Sunflower chlorotic mottle virus*, J. Phytopathol. 151 (2003) 267–273.
[34] A. Akhkha, D.C. Clarke, P.J. Dominy, Relative tolerances of wild and cultivated barley to infection by *Blumeria graminis* f. sp. *hordei* (SynErysiphe graminis f. sp. hordei). II. The effects of infection on photosynthesis and respiration, Physiol. Mol. Plant Pathol. 62 (2003) 347–354.
[35] M. Bertamini, N. Nedunchezhian, F. Tomasi, M.S. Grando, Phytoplasma [Stolbur-subgroup Bois Noir-BN)] infection inhibits photosynthetic pigments, ribulose-15-bisphosphate carboxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves, Physiol. Mol. Plant Pathol 61 (2002) 357–366.
[36] X. Zhang, L. Zhang, F. Dong, J. Gao, S.W. Galbraith, C.-P. Seng, Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*, Plant Physiol. 126 (2001) 1438–1448.
[37] X.-P. She, X.-G. Seng, J.-M. Ho, Role and relationship of nitric oxide and hydrogen peroxide in light/dark—regulated stomatal movement in *Vicia faba*, Acta Bot. Sin. 46 (2004) 1292–1300.
[38] J. He, S.K. Lee, Relationship among photosynthesis, ribulose-1,5-bisphosphate carboxylase (Rubisco) and water relations of the subtropical vegetable Chinese broccoli grown in the tropics by manipulation of root-zone temperature, Environ. Exp. Bot. 46 (2001) 119–128.
[39] J. Rahoutei, I. Garcia-Luque, M. Barón, Inhibition of photosynthesis by viral infection: effect on PS II structure and function, Physiol. Plant 110 (2000) 286–292.
[40] M. Šindelářová, L. Šindelář, TMV-RNA biosynthesis in the light-green and dark-green regions of tobacco leaves, Biol. Plant 48 (2004) 419–423.
[41] M. Havaux, K.K. Niyogi, The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 8762–8767.