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Changes in glucose-6-phosphate dehydrogenase, ribonucleases, esterases and contents of viruses in potato virus Y infected tobacco superinfected with tobacco mosaic virus

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Abstract

Effects of the superinfection with tobacco mosaic virus (TMV) on susceptible tobacco plants infected with potato virus Y (PVY) were determined. Dynamic changes in the TMV and/or PVY contents, the ribonucleases (RNases), the phosphomonoesterase (PME), the phosphodiesterase (PDE) and the glucose-6-phosphate dehydrogenase (G6P DH) activities were studied. The PVY infection caused a substantial reduction in the multiplication of TMV. The content of TMV in the PVY inoculated leaves amounts to 6 and 9 % in the PVY systemically infected leaves when compared with single TMV. Surprisingly, the challenging virus (TMV) enhanced the content of inducing virus (PVY) in the locally inoculated leaves up to 130 - 141 %. In contrast, the reduction of PVY content down to 35 - 40 % by TMV was seen in the PVY systemically infected leaves. The activities of the RNase, the PME, the PDE and the G6P DH were increased (when compared with the healthy plants) during the acute phase of single virus multiplication (PVY or TMV). The increase in the activities of the enzymes in the leaves with mixed infection was at least as high as the sum of the increases of single infections. Moreover, a higher increase than the sum was seen for G6P DH and PDE (by about 20 - 35 %).

Additional key words: challenger, inducer, *Nicotiana tabacum*, protein, PVY-RNA, resistance, rRNA, TMV-RNA.

Introduction

The phenomenon referred to as "interference" occurs when a virus is already present in cells of a host being inoculated with a second virus and infection by the second virus may then be affected by the presence of the first one. When the infection of the second virus can still be suppressed, in this case the phenomenon is known as "induced" or "acquired resistance". This "cross protection" (a systemic infection by one strain protect against challenge inoculation by other strains of the same virus) and "superinfection" (process of infection by a virus of a host previously infected by another virus) has been used extensively in practical crop protection (*e.g.* Sequeira 1984). For obvious commercial reasons, protecting strains which give mild symptoms and cause minimal loss of yield, have been favoured.

Viruses are obligate parasites and they require a suitable host for replication. Most mechanisms proposed to account for specificity in this protection assume that the events that control the outcome of the interaction of virus strains are relatively independent of the host. However, the host may play as important a role as the virus and virus strains in the events that occur in this protection. Four theories were proposed to explain this protection: utilisation and depletion of host metabolites or structures, specific sequestering of nucleic acid, involvement of coat protein (include PR-proteins and antiviral factor biosynthesis) and prevention of systemic spread of the challenge virus (Nelson *et al.* 1990, Urban *et al.* 1990, Hammerschmidt 1999, Mahmood and Rush 1999, Aguilar *et al.* 2000, Voinnet 2000). In an infected

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Abbreviations: dpi PVY - day post PVY inoculation; dpi TMV - day post TMV inoculation; G6P DH - glucose-6-phosphate dehydrogenase; PDE - phosphodiesterase; PME - phosphomonoesterase; PVY - potato virus Y; PVY/TMV - leaves infected with PVY and superinfected with TMV; RNase - ribonuclease; TMV - tobacco mosaic virus.

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host cell, virus RNA can be synthesised mainly from intermediates of the oxidative pentosephosphate pathway or from intermediates released from degraded host rRNA (Šindelářová *et al.* 1997).

Findings on the influence of virus infection on the rate of the oxidative pentosephosphate pathway have not yet been completely consistent; some authors reported a decreased rate (Bozart 1969), others unchanged rate (Takahashi 1971). However, most research workers explicitly found the increased activity of the enzymes involved in the oxidative pentosephosphate pathway (especially of both dehydrogenases), mainly in tissues surrounding local necrotic lesions (*e.g.*, Solymosy and Farkas 1962, Huth 1973). The results obtained in our earlier studies also confirmed such increased activity of the whole oxidative pentosephosphate pathway (Šindelář 1986, Šindelář *et al.* 1999a,b, Šindelářová *et al.* 1997, 1998).

Knowledge about the number of multiple forms, intracellular location and metabolic functions of the enzymes involved in the degradation of ribonucleic acids in host plant cells is not fully clear (Green 1994). The increase in the activity of ribonucleases (RNases) in plant tissues can be induced by wounding (Diener 1961, Bagi and Farkas 1967), by fungal infection (Barna *et al.* 1989), by chilling or osmotic stress (Kazmierczak and Knypl 1994) and by viral infections. Diener (1961) observed stimulation of RNases in TMV-inoculated *Datura stramonium* and in bean pod mottle virus-inoculated *Phaseolus vulgaris*, Wyen *et al.* (1972) found increased concentration of a relatively purine specific endoribonuclease in TMV-inoculated Xanthi-nc tobacco leaves, and Randles (1968) reported that the activity of one of three host RNases rose significantly at the time of rapid virus accumulation in Chinese cabbage systemically-infected with turnip yellow mosaic virus. RNases are not only typical stress enzymes, they represent one of three ways of the virus-RNA biosynthesis from precursors released from degraded host rRNA (Reddi 1963, Šindelářová *et al.* 1997, 2000a,b, 2002). Similarly, the stimulation of phosphomonoesterase (PME), phosphodiesterase (PDE) and other enzymes participating in the degradation of the host rRNA was observed (Šindelářová *et al.* 2000b, 2002).

This paper presents the results of our study of the first protection theory mentioned above: the utilisation and depletion of host metabolites, which has not yet been studied at large. It propounds the detailed study of changes in TMV content and the control enzymes of host rRNA degradation (RNases, PME, PDE) and oxidative pentosephosphate pathway (G6P DH) in PVY locally and systemically infected leaf tissue super-infected with TMV.

Materials and methods

Plant cultivation and virus inoculation: Two-month-old tobacco (*Nicotiana tabacum* L. cv. Samsun) plants grown under constant conditions in soil, at an irradiance of 100 μmol m^{-2} s^{-1} (16-h photoperiod) and average temperature of 25 °C, were used. Two leaves of the middle insertion, approximately 5 cm long, were mechanically inoculated with purified PVY (necrotic strain of potato virus Y) (Leiser and Richter 1978) at a concentration of 100 μg cm^{-3}. Corresponding leaves of control plants were mock inoculated with distilled water. These two leaves of both alternatives were named "inoculated leaves". Two upper leaves systemically infected with PVY and the corresponding healthy leaves were named "systemic leaves". The day of inoculation was designated as zero day post PVY inoculation (0 dpi PVY).

Two following types of superinfection with TMV were used in experiments:

1) "PVY inoculated/TMV inoculated on the 5th dpi PVY": The PVY inoculated leaves and the corresponding mock inoculated leaves of one half of experimental plants were on the 5th dpi PVY inoculated with purified tobacco mosaic virus (TMV, common strain, Gooding and Hebert 1967) and named "mix inoculated" (PVY+TMV) and "TMV inoculated" leaves, respectively. Similarly, the inoculated leaves of rest plants (both PVY and healthy) were mock inoculated and named "PVY inoculated" and "healthy inoculated". These inoculated leaves were used for analysis. The day of TMV-inoculation was designated as zero day post TMV inoculation or the 5th day post PVY inoculation (0 dpi TMV = 5 dpi PVY). Thus, four types of inoculated leaves were available for comparison in the first type of experiments: healthy inoculated (mock + mock inoculated); PVY inoculated (PVY + mock inoculated); TMV inoculated (mock + TMV inoculated); mix inoculated (PVY + TMV inoculated).

2) "PVY systemically infected/TMV inoculated on the 9th dpi PVY": The first two PVY systemically infected leaves (with visual symptoms of PVY) and the corresponding healthy leaves of one half of plants, were inoculated with TMV at a concentration of 100 μg cm^{-3} on the 9th dpi and named "mix systemic" and "TMV systemic". Other half of plants was mock inoculated in the same way and named "PVY systemic" and "healthy systemic". The day of TMV-inoculation was designated as zero day post TMV inoculation or the 9th day post PVY inoculation (0 dpi TMV = 9 dpi PVY). These two systemic leaves were used for analysis. Thus, four types of differently infected leaves were available for comparison in the second type of experiments: healthy systemic (mock-inoculation of two healthy systemic leaves); PVY systemic (mock inoculation of two PVY systemic leaves); TMV systemic (TMV inoculation of

two healthy systemic leaves); mix infected (TMV inoculation of two PVY systemic leaves). The leaves of 5 plants were collected on the designated day and directly homogenized in all alternatives.

Preparation of homogenate: Homogenates were prepared from the samples by grinding in a mortar with fine silica sand, 10 % (m/m) insoluble polyvinylpyrrolidone and TEMM buffer (20 mM Tris/HCl buffer, 1 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 30 mM 2-mercaptoethanol, pH 7.0) in a ratio of 1:5 (m/v). The resulting homogenate was squeezed through Miracloth and nylon sieve 100 mesh and centrifuged for 10 min at 20 000 *g*.

Preparation and storage of homogenates were carried out at 0 to 4 °C. Under these conditions the activity of the enzymes did not change for more than 5 h.

Determination of protein and chlorophyll contents and enzyme activities: Soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard, and chlorophyll according to Arnon (1949).

The ribonucleases (RNases) activity were assayed according to Šindelářová *et al.* (2000b). Amount of degraded RNA in the supernatant was determined spectrophotometrically (*Helios* type, *Unicam*, Cambridge, UK) at 260 nm. One enzyme unit (U) was defined as the amount of the enzyme causing an increase of 1.0 in the absorbance at 260 nm per hour.

Phosphomonoesterase (PME, EC 3.1.3.2) and phosphodiesterase (PDE, EC 3.1.4.1) activities were assayed using *p*-nitrophenylphosphate or bis-*p*-nitrophenylphosphate as substrates at its pH optima (5.5 and 6.0, respectively) according to Chersi *et al.* (1966).

G6P DH (EC 1.1.1.49) activity was determined spectrophotometrically, NADPH generation was monitored at 340 nm according to Šindelář *et al.* (1999a).

Enzyme activities were determined at their respective pH optima at 25 °C (with the exception 38 °C for ribonucleases).

Determination of PVY and TMV contents: PVY and TMV content were determined by the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-PVY and anti-TMV antibodies and alkaline phosphatase labeled antibodies prepared against our isolate of PVY and TMV. Virus contents were estimated on the base of calibration curve of purified PVY and TMV, respectively, using computer software described by Mančal (1987).

Statistical treatment and chemicals: The results are presented as arithmetical means (± standard deviation of mean, SDM) of 3 - 5 determinations in four independent experiments. The *t*-test and paired *t*-test was employed to characterise the differences.

Alkaline phosphatase was obtained from *Boehringer* (Heisenhofen, Germany) and all other biochemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

Results and discussion

Chlorophyll and protein contents were not significantly changed by infection with PVY, TMV and PVY/TMV, hence these data are not shown.

The multiplication of virus PVY and/or TMV, respectively, were studied in sensitive tobacco cv. Samsun spreading PVY from the inoculated leaves (local infection) to upper non-inoculated leaves (systemic infection). The PVY content (single virus) grew up gradually in the PVY inoculated leaves with maximum 4.82 ± 0.04 μg(PVY) g^{-1}(f.m.) on the 11th dpi PVY (Fig. 1*A*). In the PVY systemic leaves, the detectable amount of PVY appeared no sooner than on the 5th dpi PVY with maximum 3.44 ± 0.03 μg g^{-1}(f.m.) on the 19th dpi PVY (Fig. 1*G*).

The TMV content (single virus) reached the maximum 54.1 ± 0.5 μg g^{-1}(f.m.) in the inoculated leaves on the 6th dpi TMV (Fig. 1*A*). In the systemic leaves, the maximum of virus content was 406.2 ± 5.1 μg g^{-1}(f.m.) on the 10th dpi TMV (Fig. 1*G*).

The PVY infection caused a substantial reduction in the multiplication of TMV in both the inoculated and the systemic leaves. In the mix inoculated leaves, the total content of TMV reached 3.31 ± 0.03 μg(TMV) g^{-1}(f.m.) (Fig. 1*A*), which represented 6 % of TMV found in leaves infected with single TMV (Fig. 1*B*). In the systemic leaves, the TMV was multiplied to total 13.8 ± 1.2 μg(TMV) g^{-1}(f.m.) on the 5th dpi TMV (Fig. 1*G*), which was only 9 % of TMV determined in leaf tissues with single TMV (Fig. 1*H*). These results correspond with findings of Procházková (1970) studying the interaction of the same viruses in tobacco plants, agree with the reduction of biosynthesis of PVY in tobacco infected with PVX found by Nhu *et al.* (1982) and with a lower content of challenger virus in protoplasts of tobacco monitored by Barker and Harrison (1978) and Watts and Dawson (1980).

In contrast, the challenging virus (TMV) evoked the increase in the content of inducing virus (PVY) up to 141 % (Fig. 1*A*) with total content 5.09 ± 0.05 μg(PVY) g^{-1}(f.m.) (t = 2.776**, n = 18) in the mix inoculated leaves when compared with leaves inoculated with single PVY, which is in agreement with findings of

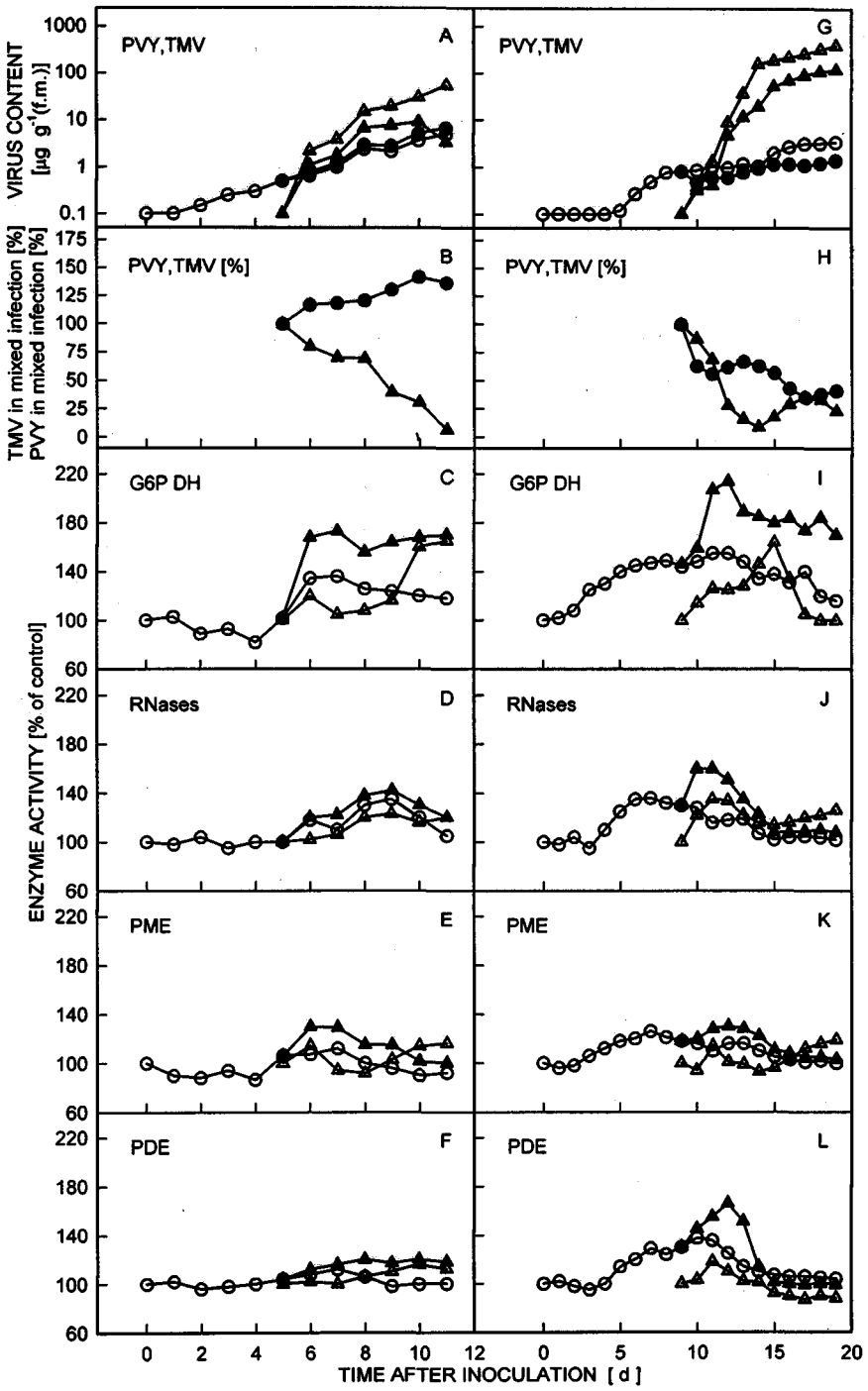

Fig. 1. *A - F (left column)* - data are from experiments where two leaves locally inoculated with PVY were inoculated with TMV on the 5th day post PVY inoculation and used for analysis; *G - L (right column)* - data are from experiments where two leaves systemically infected with PVY were inoculated with TMV on the 9th day post PVY inoculation and used for analysis. *A,G* - the content of single PVY (*open circles*), the content of PVY in mix infection (*closed circles*), the content of single TMV (*open triangles*) and the content of TMV in mix infection (*closed triangles*); *B,H* - the TMV content in mix infection/ single TMV content (*closed triangles*) and the PVY content in mix infection/ single PVY content (*closed circles*); *C,I* - the glucose-6-phosphate dehydrogenase (G6P DH); *D,J* - the ribonucleases (RNases with pH optima 5.5); *E,K* - the phosphomonoesterase (PME); *F,L* - the phosphodiesterase (PDE). Enzyme activities in single PVY infection - *open circles*, in single TMV infection - *open triangles*, and in mix infection (PVY/TMV) - *closed triangles*.

Valkonen (1992) and Goodman and Ross (1974). On the contrary, the superinfection with TMV of PVY systemic leaves caused the decrease in multiplication of PVY so that the content of PVY amounted to 35 % (Fig. 1*H*) with total content 1.09 ± 0.01 μg(PVY) g^{-1}(f.m.) (t = 4.058***, n = 22) when compared with single PVY.

Activities of RNase, PME, PDE and G6P DH were increased during the acute phase of multiplication of single virus both in the PVY and TMV infected leaves in both types of experiments.

The greatest increase in enzyme activity was observed for G6P DH: 140.2 ± 7.1 %*** in the PVY inoculated leaves (Fig. 1*C*), 155.4 ± 4.8 %*** in the PVY systemic leaves (Fig. 1*I*), 165.6 ± 5.8 %*** in the TMV systemic leaves (Fig. 1*I*) and 164.3 ± 4.8 %*** in the TMV inoculated leaves (Fig. 1*C*), when compared with the activities of the corresponding healthy leaves. The actual activities of G6P DH varied from 0.41 to 0.59 nmol g^{-1}(f.m.) s^{-1} in the healthy inoculated leaves and from 0.38 to 0.45 nmol g^{-1}(f.m.) s^{-1} in the healthy systemic leaves.

Minor increase caused by infection with single virus (TMV or PVY) was found for the activities of RNases: 123 - 135 % in the PVY or TMV inoculated leaves (Fig. 1*D*) and 131 - 136 % in the systemic leaves (Fig. 1*J*), when compared with corresponding healthy leaves. The actual activities of RNases varied from 26.41 to 48.59 U g^{-1}(f.m.) in the healthy inoculated leaves and from 22.38 to 41.45 U g^{-1}(f.m.) in the healthy systemic leaves.

The least changes were observed for the activities of PME and PDE: 112 - 114 % in the inoculated leaves (PVY or TMV) (Fig. 1*E,F*) and 119 - 137 % in the systemic leaves (PVY or TMV) (Fig. 1*K,L*). The actual activities were determined in the healthy inoculated leaves within the range of 106.4 - 112.5 μmol g^{-1}(f.m.) s^{-1} for PME and 10.8 - 13.1 μmol g^{-1}(f.m.) s^{-1} for PDE, respectively, and 88.3 - 102.5 μmol g^{-1}(f.m.) s^{-1} for PME and 8.7 - 11.3 μmol g^{-1}(f.m.) s^{-1} for PDE, respectively, in the healthy systemic leaves.

These findings are in agreement with observations of Diener (1961), Huth (1973), Randles (1968), Takahashi (1971) and with our previous experiments (Šindelář 1986, Šindelářová *et al.* 1997, 1998, 2000a,b, 2001).

In the PVY infected tobacco, the subsequent TMV inoculation induced the additional increase in the activities of studied enzymes largely within the interval of 1 - 4 dpi TMV in both types of experiments. The activities of G6P DH were enhanced up to 184 % in the mix-inoculated leaves (Fig. 1*C*) and up to 207 % in the systemic leaves (Fig. 1*I*) when compared with corresponding healthy control. The RNases were increased up to 142 % in the inoculated leaves (Fig. 1*D*) and to 160 % in the systemic leaves (Fig. 1*J*), the PME up to 130 % both in the inoculated and the systemic leaves (Figs. 1*E,K*), the PDE up to 120 % in the inoculated leaves (Fig. 1*F*) and to 166 % in the systemic leaves (Fig. 1*L*), when compared with the activities of corresponding healthy leaves. The increases in the enzymes' activities in mix infection were at least as high as the sum of the increases determined for single infections. Moreover, a higher increase than the sum was observed for G6P DH and PDE (by about 20 - 35 %) within the interval of 1 - 4 dpi TMV.

Thus, the TMV superinfection of the PVY infected tobacco induced the strong additional increase in the activities of particular enzymes connected with metabolic pathways of purine and pyrimidine nucleotide biosynthesis *in vivo*. On the basis of data of the single infections as well as the mix infection, the effectiveness of studied enzymes in replication of viral RNA can be expressed by the following sequence: G6P DH > PDE > RNases > PME.

However, the increase in G6PDH and PDE activity caused by the mix infection was higher than the sum of increases by the single infections in spite of suppress of TMV multiplication by infection with PVY. This suggests the involvement of these enzymes not only in the biosynthesis of nucleotide precursors but also in other processes, possibly connected with defence mechanisms of host. This assumption is supported by the findings of the increased activities of the studied enzymes found after treatment of tobacco leaves with benzothiodiazole, an inducer of acquired resistance. In addition, the benzothiodiazole pre-treatment also caused a substantial reduction in the TMV content of tobacco plants (Šindelářová *et al.* 2002).

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