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BRIEF COMMUNICATION

Regulation of metabolic pathways PVY-RNA biosynthesis in tobacco: host´s RNA degradation

L. ŠINDELÁŘ and M. ŠINDELÁŘOVÁ*

Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Na Karlovce 1, CZ-16000 Prague 6, Czech Republic

Abstract

Tobacco plants infected with the potato virus Y (PVY) were studied during the acute-infection period. The control enzymes of metabolic pathway of host´s RNA degradation tending to biosynthesis of PVY-RNA, its coarse/fine regulation and content of host´s RNA were monitored. Activities of ribonucleases, phosphomonoesterases and phosphodiesterases in both the crude homogenates and the partially purified enzyme preparations from the diseased leaves were markedly increased when compared to the tissues from healthy plants. The curves of enzyme activities positively correlated with the multiplication curve of the PVY and negatively correlated with the decreased contents of host´s RNA. The enzyme activity in homogenate samples did not significantly differ from the corresponding purified enzyme preparations.

Additional key words: *Nicotiana tabacum* L., phosphodiesterase, phosphomonoesterase, potato virus Y, ribonuclease, RNA content.

In an infected host cell, virus RNA can be synthesised from intermediates of the reductive pentosephosphate pathway during photosynthesis or from intermediates of the oxidative pentosephosphate pathway, which is active preferentially in the dark, or from intermediates released from degraded host RNA. These three metabolic pathways are involved in virus RNA biosynthesis, but their participation usually depends on the type of virus, host, and environmental conditions (Šindelářová *et al.* 1997).

Knowledge about the number of multiple forms, intracellular location and metabolic functions of the enzymes involved in the degradation of ribonucleic acids in plant cells is not fully clear (Farkas 1982, Green 1994). To obtain insight into the regulation of cellular RNA breakdown, extensive studies were done to localise and purify the ribonucleolytic enzymes from the plant material (Wilson 1975, Green 1994). The degradation of host´s RNA is predominantly mediated by three-types of enzymes: ribonucleases (RNases), phosphodiesterases (PDE) and phosphomonoesterases (PME) (Karlson 1981, Green 1994).

Ribonucleases (RNases) are not only typical stress enzymes, but also represent one of three ways of the virus-RNA biosynthesis from precursors released from degraded host RNA. Diener (1961) observed stimulation of RNases in TMV-inoculated *Datura stramonium* and in BPMV-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 TYMV. The similar results were found also by Reddi (1959, 1963), Cheo (1971), Šindelář *et al.* (1990), Šindelářová *et al.* (2000, 2002), Šindelářová and Šindelář (2003, 2003/4, 2004).

This paper presents the study of regulative mechanisms in the intensities of pathways of host´s RNA degradation found in tobacco leaf tissues upon potato virus Y infection.

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Abbreviations: PDE - phosphodiesterase; PME - phosphomonoesterase; RNases - ribonucleases; PVY - potato virus Y.
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* Corresponding author; fax: (+420) 224 310 113; e-mail: sindelarova@ueb.cas.cz

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 bottom 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. The day of inoculation was designated as zero day post PVY inoculation (0 dpi PVY). The two systemically infected leaves of 10 plants were collected on the designated day and directly homogenized in all alternatives.

Crude homogenates ("homogenate") 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*.

The partially purified enzyme preparation ("purificate") was obtained from 3 cm^3 of the crude homogenate by means of quantitative precipitation with ammonium sulphate. The fraction between 0.2 and 0.85 salt saturation was separated by centrifugation (20 000 *g* for 10 min), the precipitate resuspended in 2.5 cm^3 of TEMM buffer, desalted by centrifugation method through *Sephadex G-25 Fine* and made up to the original volume of 3 cm^3 to enable comparison of the partially purified preparation with the crude homogenate on a volume basis. Fractions below 0.20 and above 0.85 salt saturation did not contain measurable enzyme activity. Preparation and storage of both the crude homogenates and the purified enzyme preparations was carried out at 0 - 4 °C. Under these conditions, the activities of the enzymes did not change for more than 5 h.

RNA content in leaf samples was determined according to the method of Nishimura *et al.* (1976). PVY content were determined by the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-PVY antibodies and alkaline phosphatase labeled antibodies raised against our isolate of PVY. Virus contents were estimated on the base of calibration curve of purified PVY using computer software described by Mančal (1987).

The activities of ribonucleases (RNases) were assayed according to Šindelářová *et al.* (2000). Amount of degraded RNA in the supernatant was determined spectrophotometrically at 260 nm at its pH optima (5.5, resp. 7.0). 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 phospho-diesterase (PDE; EC 3.1.4.1) activities were assayed using *p*-nitrophenylphosphate or bis-*p*-nitrophenylphosphate as substrates at its pH optima (5.5, resp. 6.0) according to Chersi *et al*. (1966). Enzyme activities were determined at 38 °C.

The results are presented as arithmetical means (± standard deviation of mean, SD) 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).

The content of RNA (shown as percentages of healthy control) negatively linearly correlate with the multiplication curve of PVY (r = -0,956*** for n = 36) (Fig. 1*A*,*B*).

The activities of RNases, PME and PDE were estimated in both "homogenates" and "purificates". Activities of RNases with pH optimum 5.5 ranged from 18 to 33 U g^{-1}(f.m.) in "homogenates" and from 16 to 46 U g^{-1}(f.m.) in "purificates", RNases with pH optimum 7.0 ranged from 11 to 19 U g^{-1}(f.m.) and from 9 to 17 U g^{-1}(f.m.), respectively. The range of PME activities was 443 - 458 nmol g^{-1}(f.m.) min^{-1} in "homogenates" and 410 - 422 nmol g^{-1}(f.m.) min^{-1} in "purificates", and PDE 8 - 10 nmol g^{-1}(f.m.) min^{-1} in "homogenates" and 7 - 9 nmol g^{-1}(f.m.) min^{-1} in "purificates", respectively.

The RNase, PME and PDE activities of diseased tissues (Fig. 1*C-F*) (shown as percentages of healthy

Table 1. Correlations between enzyme activities and PVY content or enzyme activities and host´s RNA content in tobacco infected with PVY. Paired *t*-test was used to test hypothesis about differences between enzyme activity found in "homogenate" and "purificate". *** - the difference is statistically significant at $P < 0.001$ (data without symbols are not statistically significant).

	Enzyme vs. PVY		Enzyme vs. RNA		"Homogenate" vs. "purificate"	
	r	*n*	*r*	*n*	*t*	*n*
RNases pH = 5.5	0.968***	26	-0.916***	26	1.067	34
RNases pH = 7.0	0.954***	28	-0.956***	28	1.126	32
PME	0.943***	32	-0.986***	32	1.021	28
PDE	0.983***	30	-0.978***	30	1.118	26

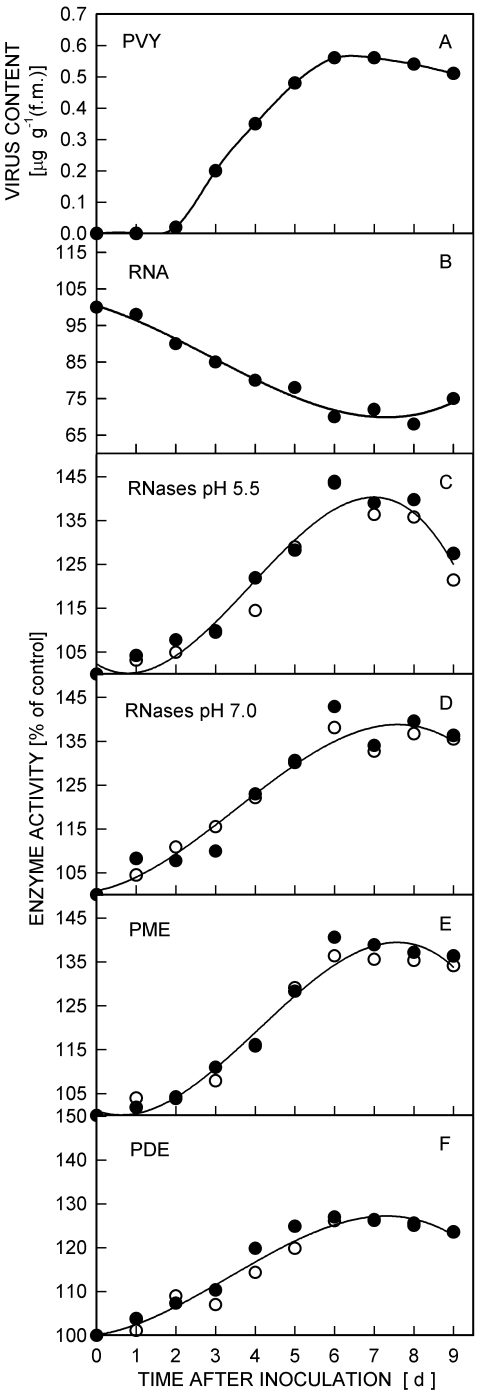

Fig. 1. The PVY (*A*) and host´s RNA content (*B*), the ribonucleases (RNases with pH optima 5.5 (*C*) and 7.0 (*D*), the phosphomonoesterases (PME) (*E*) and the phosphodiesterases (PDE) (*F*) activities in the systemically infected leaves of *Nicotiana tabacum* L. cv. Samsun. The PVY content is given in μg PVY g^{-1}(f.m.), the RNA content and enzymes activities are expressed in % of the healthy control. The closed marks represent the activities of enzymes in "homogenate", the open marks in "purificate" in Figs. *C-F*. Regression curves are calculated using values both of "homogenate" and of "purificate" with regards to the fact that no significant differences were found between both sets.

control) were markedly increased over the experimental period, the curves of activities positively correlated with the multiplication curve of the PVY and negatively correlated with the decreased content of host´s RNA (Fig. 1, Table 1).

Similarly, the activities of RNase as well as PME and PDE were twice as high in infected chloroplasts of PVY infected tobacco when compared with healthy chloroplasts (Šindelářová *et al.* 2000). This agrees with the findings of Oxelfelt (1971), Fraser (1972) and Suzuki and Taniguchi (1973) who described a strong degradation of chloroplast RNA in the infected tissue in contrast to much lower degradation of the cytoplasmic RNA, particularly in the last period of infection.

The increase in the RNase, PME and PDE activities of virus-infected tobacco leaves were observed both in the crude homogenates and in the partially purified enzyme preparations with low molecular mass effectors removed. The statistical evaluation by paired *t*-test did not show significant differences between enzyme activity in homogenate and corresponding purified enzyme preparation (Table 1).

In conclusion, the activities of RNases, PME, PDE are markedly increased in virus-infected tissues over an acute-infection period both in crude homogenate and after partial purification when compared with healthy controls. The enzyme activity in homogenate samples did not significantly differ from the corresponding purified enzyme preparations. This implies that the increases in the studied enzymes are a result of their enhanced *de novo* synthesis in infected tobacco and not the result of fine regulation by low molecular mass effectors.

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