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BRIEF COMMUNICATION

Changes in key enzymes of viral-RNA biosynthesis in chloroplasts from PVY and TMV infected tobacco plants

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Abstract

Effects of the infection with tobacco mosaic virus (TMV) and potato virus Y (PVY) on chloroplasts from susceptible tobacco plants were determined. Changes in ribonucleases (RNases), phosphomonoesterase (PME), phosphodiesterase (PDE), glucose-6-phosphate dehydrogenase (G6P DH), 6-phosphogluconate dehydrogenase (6PG DH), glucokinase (GK), and fructokinase (FK) activities in thylakoid/envelope and stroma fractions were studied. Slight increase in the activities of PME, PDE, G6P DH and 6PG DH of thylakoid/envelope fraction as well as of RNases, PME, PDE, G6P DH, 6PG DH, GK and FK of stroma fraction was found in chloroplasts isolated from leaf tissues infected with PVY. Infection with TMV produced higher increase in enzymes activities in chloroplasts; especially, PME, G6P DH and 6PG DH in fraction of thylakoid/envelope, and RNases, PME, PDE, G6P DH, 6PG DH, and GK in stroma fraction.

Additional key words: chlorophylls, *Nicotiana tabacum* L. cv. Samsun, protein, RNA.

Viruses are obligate parasites that require a suitable host for their replication. Virus RNA can be synthesised from the intermediates of reductive pentosephosphate pathway during photosynthesis, from the intermediates of oxidative pentosephosphate pathway, and also from nucleotide precursors degraded from host RNA. In an infected host cell of tobacco, virus RNA can be synthesised mainly from the intermediates of the oxidative pentosephosphate pathway or from the 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 quite consistent; some authors reported a decreased rate (*e.g.* Bozart 1969), other unchanged rate (*e.g.* Takahashi 1971). However, most researchers 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, Šindelář and Šindelářová 1987a,b, 2002a,b, Šindelářová *et al*. 1997, Šindelářová and Šindelář 2003/4, 2004) and the increase of both dehydrogenases directly in chloroplasts isolated from protoplast of infected tobacco (Šindelář and Šindelářová 2002a).

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), fungal infection (Barna *et al*. 1989), chilling or osmotic stress (Kazmierczak and Knypl 1994), and 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)

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Abbreviations: dpi - day post virus inoculation; FK - fructokinase; GK - glucokinase; G6P DH - glucose-6-phosphate dehydrogenase; PDE - phosphodiesterase; 6PG DH - 6-phosphogluconate dehydrogenase; PME - phosphomonoesterase; PVY - potato virus Y; RNases - ribonucleases; TMV - tobacco mosaic virus.

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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, but they also represent one of three ways of the virus-RNA biosynthesis from precursors released from degraded host rRNA (Reddi 1963, Šindelář *et al*. 1990, Šindelářová *et al*. 1997, 2000). Similarly, the stimulation of phosphomonoesterase (PME), phosphodiesterase (PDE), and other enzymes participating in the degradation of the host rRNA was observed in tissues and directly in chloroplasts (Šindelářová *et al.* 2000).

Participation of subcellular compartments in biosynthesis of various plant viruses is not fully explained. TMV virions were determined in vacuoles and mitochondria (Gargouri and Haenni 1990), TMV-replicase in cytoplasm ("viroplasm", Hills *et al.* 1987), Gargouri and Haenni (1990) expected biosynthesis of PVY in cytoplasm, Gadh and Hari (1986) found ds-RNA of tobacco etch virus (other member of the genus *Potyvirus*) in chloroplasts. In previous studies, the increase in key enzymes of the oxidative pentose-phosphate pathway (Šindelář and Šindelářová 2002a) and enzymes involved in release of intermediates from degraded host RNA (Šindelářová *et al.* 2000) was observed in chloroplasts isolated from protoplast of PVY infected tobacco.

To determine localization of isoenzymes in a chloroplast connected with virus infection we presented detailed study of changes in the key enzymes of host rRNA degradation (RNases, PME and PDE), oxidative pentosephosphate pathway (G6P DH / 6PG DH system) and glycolysis / oxidative pentosephosphate pathway (GK and FK) in chloroplasts of PVY and TMV systemically infected leaf tissue.

Two-month-old tobacco (*Nicotiana tabacum* L. cv. Samsun) 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) or TMV (common strain of tobacco mosaic virus) (Gooding and Hebert 1967) at a concentration of 100 μg cm^{-3}. Corresponding leaves of control plants were mock inoculated with distilled water. Two upper leaves infected systemically and the corresponding healthy leaves were used for chloroplasts isolation. The day of inoculation was designated as zero day post PVY inoculation (0 dpi).

Chloroplasts were prepared from systemically infected leaves collected at the end of dark period on the 10^{th} dpi by homogenisation of 30 g leaf tissue with 130 cm^3 of isolation medium contained 50 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 330 mM sorbitol and 30 mM 2-mercaptoethanol, pH 7.8 (IM), and 40 % Percoll cushion centrifugation according to Robinson and Barnett (1988). The isolated chloroplasts were contaminated with mitochondria under 1.8 %. Intact chloroplasts were twice washed with medium A (IM except EDTA), resuspended in medium B (medium A except sorbitol) and broken by repeated suction (20×) in tip of digital Finnpipette (*Labsystems*, Finland). Broken chloroplasts were centrifuged at 40 000 g for 20 min, supernatant (stroma) tapped, pellet resuspended in medium B and centrifuged on 40 % Percoll cushion in order to remove starch (pellet) from thylakoid/envelope fraction. The stroma fraction was used for the determination of activities of the enzymes, and protein and chlorophyll contents; the thylakoid/envelope fraction was solubilized with 1 % Triton X-100 at 0° C for 1 h in dark. Preparation and storage of chloroplasts were carried out at 0 to 4 °C in dark. Under these conditions, the activity of the enzymes did not change for more than 6 h.

Soluble protein content was determined according to Bradford (1976) and Miller (1959) using bovine serum albumin (BSA), or BSA with 1 % Triton X-100 as a standard. Chlorophyll content was determined in acetone extracts by spectrophotometer (*Helios* type, *Unicam*, Cambridge, UK) according to Arnon (1949).

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

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 their pH optima (5.5 and 6.0, respectively) according to Chersi *et al*. (1966).

Glucose-6-phosphate dehydrogenase (G6P DH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PG DH, EC 1.1.1.44) activities were determined spectrophotometrically, NADPH generation was monitored at 340 nm according to Šindelář *et al*. (1999a).

Glucokinase (GK, EC 2.7.1.1) was determined spectrophotometrically at 340 nm on the basis of $NADP^+$ reduction in the presence of an excess of glucose-6-phosphate dehydrogenase and ATP. Fructokinase (FK, EC 2.7.1.4) was determined similarly; the assay mixture contained fructose and in addition of glucose-phosphate isomerase (Šindelář *et al.* 1999b).

Enzyme activities were determined at their respective pH optima at 25 °C (with the exception of 38 °C for ribonucleases, PME, and PDE). Triton X-100 (1 % concentration) used for solubilization of membranes did not change the activities of studied enzymes.

The results are presented as arithmetical means ± standard error of mean (SE) of 3 - 5 determinations in three to six independent experiments. The *t*-test and paired *t*-test was employed to characterise the differences. Biochemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

Chlorophyll content as well as protein content were

Table 1. The activities of G6P DH, 6PG DH, PME, PDE, GK, FK [nmol mg^{-1}(protein) min^{-1}] and RNases [mU mg^{-1}(protein)] in the chloroplast fractions (thylakoid/envelope and stroma) isolated from the healthy (H), PVY-infected (PVY), and TMV-infected (TMV) *Nicotiana tabacum* L. cv. Samsun leaves. Means ± SE, * - the difference is statistically different at $0.01 \leq P < 0.05$, ** - $P < 0.01$ and *** - $P < 0.001$ (data without symbols are not statistically different), ND - not detected. The data in parentheses are expressed in % compared to the healthy mock-inoculated control (100 %).

Enzyme		Thylakoid/envelope		Stroma	
G6P DH	H	0.62 ± 0.05	(100.0)	1.51 ± 0.12	(100.0)
	PVY	0.72 ± 0.06*	(116.1)	1.87 ± 0.16**	(123.8)
	TMV	0.84 ± 0.07***	(135.5)	2.45 ± 0.22***	(162.3)
6PG DH	H	12.08 ± 1.08	(100.0)	33.64 ± 2.65	(100.0)
	PVY	13.11 ± 1.12	(108.5)	36.49 ± 2.92	(108.5)
	TMV	15.26 ± 1.48**	(126.3)	57.12 ± 4.93***	(169.8)
RNases pH 5.5	H	ND		3.62 ± 0.29	(100.0)
	PVY	ND		3.75 ± 0.29	(103.6)
	TMV	ND		5.76 ± 0.44***	(159.1)
RNases pH 7.0	H	ND		5.51 ± 0.43	(100.0)
	PVY	ND		6.85 ± 0.54**	(124.3)
	TMV	ND		12.07 ± 1.08***	(219.1)
PME	H	1.54 ± 0.11	(100.0)	10.12 ± 1.05	(100.0)
	PVY	1.61 ± 0.14	(104.5)	10.56 ± 0.91	(104.3)
	TMV	2.17 ± 0.19***	(140.9)	18.39 ± 1.63***	(181.7)
PDE	H	1.14 ± 0.09	(100.0)	2.46 ± 0.18	(100.0)
	PVY	1.31 ± 0.11*	(114.9)	2.64 ± 0.22	(107.3)
	TMV	1.41 ± 0.12**	(123.7)	2.73 ± 0.24	(111.0)
GK	H	ND		0.97 ± 0.08	(100.0)
	PVY	ND		1.16 ± 0.09*	(119.6)
	TMV	ND		1.57 ± 1.04***	(161.9)
FK	H	ND		7.46 ± 0.61	(100.0)
	PVY	ND		8.75 ± 0.69*	(117.3)
	TMV	ND		9.28 ± 0.83**	(124.4)

not changed by PVY and TMV infections, respectively (results not shown). Similarly, chlorophyll *a/b* ratio was about 2.32 for all alternatives. This indicates that chloroplasts were not impaired during acute phase of infection (10 dpi).

Slight increase in the activities of PME, PDE, G6P DH and 6PG DH of the thylakoid/envelope fraction as well as of RNases (pH optima 5.5 and 7.0), and PME, PDE, G6P DH, 6PG DH, GK and FK activities of the stroma fraction was found in the chloroplasts isolated from leaf tissue infected with PVY when compared with the healthy control (Table 1). In contrast, much more increase in the activities of PME, PDE, G6P DH and 6PG DH of the thylakoid/envelope fraction and of RNases (pH optima 5.5 and 7.0), PME, PDE, G6P DH, 6PG DH, GK and FK of the stroma fraction was observed in the chloroplasts of TMV infected leaves (Table 1). No measurable activities of GK, FK and RNases (pH optima 5.5 and 7.0) were found in the thylakoid/envelope fraction of the chloroplasts.

Gargouri and Haenni (1990) considered the PVY synthesis in cytoplasm, but Gadh and Hari (1986) found ds-RNA of tobacco etch virus (another member of the genus *Potyvirus*) in chloroplasts. In this case, the increase of key enzymes of the oxidative pentosephosphate pathway and enzymes involved in release of intermediates from degraded host RNA found in chloroplasts could indicate that the chloroplast has a role in biosynthesis of intermediates of PVY-RNA.

TMV is considered to be synthesised in vacuoles and mitochondria (Gargouri and Haenni 1990), and on cytopathological structures “viroplasms” in cytoplasm (Hills *et al.* 1987). Similarly to PVY, the TMV infection triggered the increase of key enzymes of the oxidative pentosephosphate pathway and enzymes involved in release of intermediates from degraded host RNA in chloroplasts. Further, the presented data indicate the increase of GK and FK (key enzymes of glycolysis), which phosphorylate monosacharides derived from free, storage and transport saccharides needed both for glycolysis and oxidative pentosephosphate pathway. The increase in the activities of those hexokinases was seen in chloroplasts of PVY infected tobacco as well. Connection of the studied enzymes with virus RNA biosynthesis stays unclear. Detail analysis of RNAs specific for biosynthesis of TMV and PVY in chloroplasts is required.

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