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Potato virus-Y multiplication in susceptible tobacco cultivar and transgenic breeding line producing coat protein mRNA

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Abstract

Changes in ribonucleases (RNases) and glucose-6-phosphate dehydrogenase (G6P DH) activities, their content and subcellular localisation were studied in relation to virus multiplication in susceptible (cv. Samsun) or resistant (transgenic breeding line NCTG 83) tobacco plants infected with the potato virus Y^N (necrotic strain of PVY). Activities of RNases and G6P DH from diseased susceptible tobacco plants were markedly increased during the experimental period and significantly correlated with the multiplication curve of the PVY^N. In contrast, the activities of RNases and G6P DH were not changed after PVY inoculation of resistant breeding line NCTG 83 producing the CP mRNA of PVY. Changes in the content and in the subcellular localisation of RNases and G6P DH isozymes were also determined in mesophyll protoplasts isolated from healthy as well as PVY^N infected plants of both cultivars by differential centrifugation of broken protoplasts on day eight post inoculation (the culmination of multiplication curve of PVY and enhanced activity of both enzymes). The chloroplasts fraction from infected protoplasts showed an enhanced content of RNases (192.4 % when compared with that from healthy control ones), and of G6P DH (174.4 %). The cytosol fraction from infected protoplasts contained slightly enhanced levels of G6P DH (117.4 %) and considerably enhanced levels of RNases (141.7 %). No significant differences in the activities, contents and subcellular localisation of RNases and/or G6P DH isozymes were observed in the resistant line NCTG 83. This is in accordance with no detectable contents of PVY.

Additional key words: glucose-6-phosphate dehydrogenase, *Nicotiana tabacum* L., ribonuclease.

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Abbreviations: BPMV - bean pod mottle virus; CP - coat protein; PVY - potato virus Y; TMV - tobacco mosaic virus; TYMV - turnip yellow mosaic virus.

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Introduction

In an infected host cell, virus RNA can be synthesised from intermediates released from degraded host rRNA or from intermediates of the oxidative pentosephosphate pathway which is active preferentially in the dark, or initially from intermediates of the reductive pentosephosphate pathway during photosynthesis. 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).

With respect to the degradation of ribonucleic acids in plant cells, the precise number of multiple forms, intracellular location and metabolic functions of the enzymes involved is not clear (Farkas 1982). To obtain insight into the regulation of cellular RNA breakdown, extensive studies have been done to localise and purify the ribonucleolytic enzymes from the plant material (Green 1994).

An increase in activity of ribonucleases (RNases) in plant tissues may be induced by wounding (Diener 1961), by infection of fungi (Barna *et al.* 1989), by chilling or osmotic stress (Kazmierczak and Knypl 1994), or by viral infections. 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 pure 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. RNases are not only typical stress enzymes, they represent one of three ways of virus-RNA biosynthesis from precursors released from degraded host rRNA (Cheo 1971, Reddi 1963, Šindelář *et al.* 1990, Šindelářová *et al.* 1997).

The oxidative pentosephosphate pathway metabolises glucose-6-phosphate to ribose-5-phosphate necessary for *de novo* biosynthesis of purine and pyrimidine nucleotides of viral RNA. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$oxidoreductase, EC 1.1.1.49., G6P DH) plays an important role in the regulation of the pathway which together with the 6-phosphogluconolactonase hydrolysing 6-phospho-D-gluconate lactone to 6-phospho-D-gluconate form a suitable irreversible enzyme system (Turner and Turner 1980). Close connections between increased activity of G6P DH and the contents of viruses in tissues was reported, *e.g.*, by Merrett and Sunderland (1967) and Huth (1973), and the results obtained in our earlier studies also confirmed increased activity of the whole oxidative pentosephosphate pathway including ribosephosphate pyrophosphokinase (Šindelář 1986, Šindelář and Šindelářová 1987a,b, Burketová 1995, Šindelářová *et al.* 1997).

The relation between the above mentioned metabolic pathways and the degree of host susceptibility to virus-RNA multiplication is not quite clear, and in transgenic plants have not been studied. For this reason, in this paper we present the study of dynamic changes in RNases and G6P DH activities, and contents and subcellular localisation of their isozymes in connection with susceptibility of two tobacco genotypes to PVY^N multiplication.

Materials and methods

Tobacco plants (*Nicotiana tabacum* L.) cv. Samsun and breeding line NCTG 83 were grown on glass beads with Hoagland nutrient solution under an irradiance of 60 μmol m^{-2} s^{-1} (16-h photoperiod) and a temperature of 25 °C. Lower leaves, approximately 5 cm long, were mechanically inoculated with purified potato virus Y^N (necrotic strain of PVY, Leiser and Richter 1978) at a concentration of 100 μg cm^{-3}; corresponding leaves of control plants were treated with distilled water. The day of inoculation was designated as day zero. The directly inoculated leaves were used for enzyme determination. Transgenic breeding line NCTG 83 possesses a transgenic system encoding PVY coat protein (CP). The CP-RNA transcript is not translated, however, because of a stop codon inserted immediately downstream from the translation initiation site. Therefore, the plants produce the CP mRNA but do not synthesise coat protein (Smith *et al.* 1994). The plants are completely resistant to PVY.

Crude homogenates were prepared by grinding samples of leaf tissues 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 $MgCl_2$, 30 mM 2-mercaptoethanol, pH 7.0) in a ratio of 1:5 (m/v). The resulting homogenate was squeezed through *Miracloth* and a 100 mesh nylon sieve and centrifuged at 20 000 *g* for 10 min. All procedures were performed at 0 to 4 °C.

Protoplasts were prepared from leaf strips according to Šindelářová and Šindelář (1994), 8 d post inoculation. The palisade parenchyma protoplasts were separated by centrifugation in discontinuous gradient by the method of Fannin and Shaw (1987). The number of protoplasts was determined in a haemocytometer and the number of viable protoplasts by staining with Methylene Blue according to Hooley and McCarthy (1980). Protoplasts were passed three times through a sieve with 20 μm meshes which resulted in their complete disintegration as monitored by light microscopy. The cell organelle suspension, designated as the crude homogenate S_0, was used for the determination of protein, chlorophyll content, phosphoenol-pyruvate carboxylase (PEPC), G6P DH, and RNase activities. The remainder of the cell organelle suspension was centrifuged for 5 min at 1 000 *g*. The pellet (P_1) containing the substantial part of the chloroplasts, was resuspended in TEMM medium. The supernatant was centrifuged for 15 min at 15 000 *g* and the supernatant (S_{15}) and the pellet (P_{15}) were obtained. The P_{15} pellet was resuspended in TEMM medium. All steps of cytosol and organelle preparation were carried out at 0 to 4 °C.

PVY content was determined by the quantitative DAS-ELISA method, the percentage of inoculated protoplasts being determined by an immunoenzymatical method according to Šindelář and Šindelářová (1994).

Protein content was determined according to Bradford (1976) (prior to addition of Triton X-100 to fractions) using bovine serum albumin as a standard, and chlorophyll content (marker of chloroplasts) according to Arnon (1949).

G6P DH (EC 1.1.1.49.) activities were determined spectrophotometrically (Šindelář 1986). The results obtained for G6P DH activities have been expressed as the half-level of nanomoles of the generated NADPH, with respect to a high activity

of phosphogluconate dehydrogenase (the following enzyme in the pathway which also reduced $NADP^+$) in homogenates. Determining the activity of these enzymes, Triton X-100 (final concentration 0.05 %) was added to all fractions, and the activity was determined after 30 min, when all chloroplasts were already completely disintegrated. Triton X-100 did not affect the activity of the enzymes.

The ribonuclease (RNase) activity was determined according to Cheo (1971). The enzyme unit (U) was defined as the amount of enzyme causing unit increase at absorbance of 260 nm.

Phosphoenolpyruvate carboxylase activity (EC 4.1.1.31, marker of cytosol) was determined according to Downton and Slatyer (1971). Enzyme activities were measured at 25 °C.

The results in Tables and Fig. 1 represent as means ± standard deviations of means of 3 - 7 determinations in four independent experiments. The *t*-test was employed to characterise the differences.

Results and discussion

The multiplication curve of PVY^N in leaves of the susceptible cultivar Samsun culminated on day eight post inoculation (Fig. 1). The content of PVY^N was not detectable even after 24 d in leaves of resistant tobacco line NCTG 83. Protein and

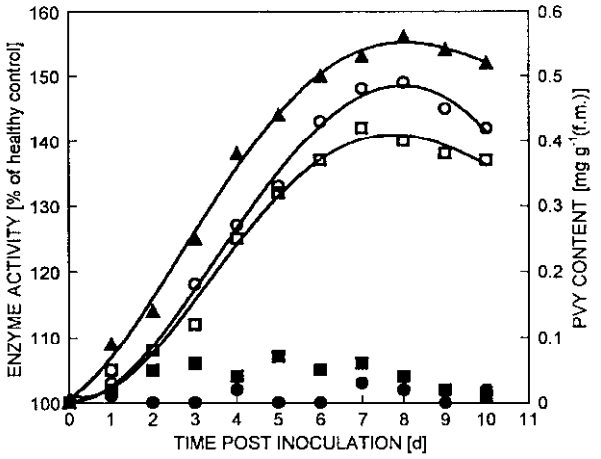

Fig.1. Multiplication curve of PVY in cv. Samsun (*triangles*), and ribonucleases (*squares*) and glucose-6-phosphate dehydrogenase (*circles*) activities in crude homogenate from tobacco leaves of susceptible cv. Samsun (*open symbols*) and resistant breeding line NCTG 83 (*closed symbols*) infected with PVY^N.

chlorophyll content in crude homogenates from virus-infected plant tissues of cv. Samsun and NCTG 83 did not significantly differ from that found in healthy tissues (Table 1). Activities of RNases and G6P DH from diseased cv. Samsun

Table 1. Protein and chlorophyll contents [mg g^{-1}(f.m.)] and activities of glucose-6-phosphate dehydrogenase (G6P DH) [nmol g^{-1}(f.m.) s^{-1}] and ribonucleases (RNases) [U mg^{-1}(proteins)] in homogenate from susceptible (cv. Samsun) and resistant (transgenic breeding line NCTG 83) tobacco leaves, healthy (H) and systemically infected with potato virus Y necrotic strain (PVY) during 10 d post inoculation (DPI).

DPI		Samsun proteins	chlorophyll	G6P DH	RNases	NCTG 83 proteins	chlorophyll	G6P DH	RNases
0		3.16 ± 0.29	0.84 ± 0.006	0.576 ± 0.020	34.31 ± 0.82	3.68 ± 0.31	0.98 ± 0.007	0.670 ± 0.020	39.90 ± 0.96
1	H	2.94 ± 0.22	0.63 ± 0.005	0.633 ± 0.022	34.41 ± 1.04	3.42 ± 0.26	0.73 ± 0.006	0.744 ± 0.023	40.01 ± 1.13
	PVY	2.80 ± 0.19	0.58 ± 0.005	0.658 ± 0.025	34.75 ± 1.18	3.42 ± 0.29	0.73 ± 0.006	0.706 ± 0.020	42.01 ± 1.27
2	H	2.96 ± 0.24	0.56 ± 0.005	0.680 ± 0.025	34.59 ± 1.11	3.44 ± 0.28	0.65 ± 0.006	0.791 ± 0.054	40.22 ± 1.56
	PVY	2.90 ± 0.21	0.56 ± 0.005	0.777 ± 0.054	37.36 ± 1.46	3.27 ± 0.12	0.66 ± 0.006	0.775 ± 0.049	42.63 ± 1.72
3	H	3.28 ± 0.32	0.49 ± 0.004	0.679 ± 0.024	34.86 ± 1.21	3.81 ± 0.33	0.57 ± 0.005	0.789 ± 0.043	40.53 ± 1.17
	PVY	3.20 ± 0.31	0.49 ± 0.004	0.801 ± 0.025**	39.04 ± 1.51	3.50 ± 0.24	0.59 ± 0.005	0.789 ± 0.043	43.37 ± 1.76
4	H	3.80 ± 0.35	0.46 ± 0.003	0.637 ± 0.024	35.17 ± 1.19	4.43 ± 0.41	0.53 ± 0.004	0.741 ± 0.035	40.89 ± 1.88
	PVY	3.54 ± 0.33	0.45 ± 0.003	0.843 ± 0.043**	43.95 ± 1.82**	3.93 ± 0.33	0.55 ± 0.005	0.753 ± 0.033	44.16 ± 1.92
5	H	4.18 ± 0.39	0.41 ± 0.002	0.612 ± 0.022	35.39 ± 1.27	4.86 ± 0.41	0.48 ± 0.004	0.711 ± 0.036	41.15 ± 1.67
	PVY	3.97 ± 0.36	0.41 ± 0.003	0.882 ± 0.049***	46.72 ± 1.93**	4.18 ± 0.37	0.49 ± 0.004	0.697 ± 0.031	43.62 ± 2.06
6	H	4.35 ± 0.42	0.38 ± 0.003	0.679 ± 0.027	35.72 ± 1.22	5.06 ± 0.47	0.44 ± 0.003	0.789 ± 0.035	41.53 ± 1.90
	PVY	4.18 ± 0.39	0.38 ± 0.002	0.969 ± 0.061***	49.29 ± 2.10***	4.30 ± 0.36	0.46 ± 0.003	0.789 ± 0.032	44.44 ± 2.28
7	H	4.58 ± 0.43	0.35 ± 0.002	0.896 ± 0.041	36.65 ± 1.31	5.33 ± 0.50	0.41 ± 0.003	1.042 ± 0.044	42.62 ± 1.73
	PVY	4.49 ± 0.42	0.34 ± 0.001	1.414 ± 0.064***	52.05 ± 2.18***	4.46 ± 0.38	0.43 ± 0.003	1.061 ± 0.033	46.03 ± 2.68
8	H	4.63 ± 0.44	0.35 ± 0.002	0.952 ± 0.053	37.67 ± 1.39	5.38 ± 0.52	0.40 ± 0.002	1.107 ± 0.038	43.80 ± 1.24
	PVY	4.52 ± 0.43	0.34 ± 0.001	1.418 ± 0.115***	52.74 ± 2.17***	5.00 ± 0.43	0.40 ± 0.002	1.085 ± 0.036	46.43 ± 2.96
9	H	4.68 ± 0.41	0.35 ± 0.002	1.212 ± 0.094	38.84 ± 1.44	5.44 ± 0.52	0.40 ± 0.001	1.410 ± 0.104	45.16 ± 1.33
	PVY	4.64 ± 0.40	0.33 ± 0.002	1.761 ± 0.110***	52.82 ± 2.26**	5.61 ± 0.54	0.39 ± 0.002	1.339 ± 0.119	46.97 ± 2.66
10	H	4.75 ± 0.45	0.35 ± 0.001	1.407 ± 0.119	40.67 ± 1.63	5.52 ± 0.53	0.40 ± 0.002	1.636 ± 0.120	47.29 ± 1.16
	PVY	4.72 ± 0.44	0.32 ± 0.001	1.998 ± 0.110***	55.31 ± 2.35**	5.96 ± 0.57	0.38 ± 0.002	1.723 ± 0.140	48.24 ± 2.04

* $0.01 \leq P < 0.05$; ** $0.001 \leq P < 0.01$; *** $P < 0.001$; data without symbols are not statistically significant

Table 2. Subcellular localization of glucose-6-phosphate dehydrogenase and ribonucleases in mesophyll protoplasts from susceptible (cv. Samsun) and resistant (transgenic breeding line NCTG 83) tobacco leaves, healthy and inoculated with potato virus Y (necrotic strain) 8 d post inoculation.

Fractions	Samsun Proteins	Chlorophyll	PEPC	G6P DH	RNases	NCTG 83 Proteins	Chlorophyll	PEPC	G6P DH	RNases
healthy plants										
S_0	339.7±3.92	77.4±0.83	379.2±24,3	43.3±0.6	94.9±0.9	90.2±1.82	73.5±0.64	379.4±24.0	55.2±0.7	90.3±0.8
	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)
P_1	123.7±1.12	72.4±0.66	0	7.5±0.3	6.6±0.1	74.9±0.71	71.4±0.69	0	10.4±0.3	5.96±0.05
	(36.4)	(93.5)	(0.0)	(15.1)	(7.0)	(39.4)	(97.2)	(0.0)	(17.2)	(6.6)
P_{15}	9.9±0.13	1.5±0.01	8.5±1.1	2.4±0.1	10.6±0.1	3.4±0.05	1.25±0.02	11.0±1.3	2.4±0.1	9.12±0.02
	(2.9)	(1.9)	(2.1)	(3.8)	(11.2)	(1.8)	(1.7)	(2.9)	(2.6)	(10.1)
S_{15}	214.0±2.64	0	356.2±23.9	35.0±0.4	79.6±0.8	109.2±0.96	0	376.3±22.8	44.4±0.5	73.3±0.07
	(63.0)	(0.0)	(94.0)	(79.8)	(83.9)	(57.4)	(0.0)	(99.2)	(80.9)	(81.2)
Σ	(102.3)	(95.4)	(96.1)	(98.7)	(102.1)	(98.6)	(98.9)	(102.1)	(100.7)	(97.9)
infected plants										
S_0	337.7±3.85	59.2±0.63	290.5±23.4	52.4±0.8	130.0±1.4	186.2±1.67	60.2±0.55	373.4±28.6	57.5±0.9	81.77±0.08
	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)	(100.0)
P_1	108.1±1.22	53.0±0.55	0	11.5±0.3	12.7±0.5	75.2±0.71	59.1±0.44	0	11.4±0.4	5.32±0.04
	(32.0)	(89.5)	(0.0)	(22.0)	(9.8)	(40.4)	(98.2)	(0.0)	(18.9)	(6.5)
P_{15}	9.5±0.11	1.1±0.01	8.5±0.9	2.0±0.1	12.6±0.4	3.3±0.01	1.08±0.02	11.3±1.1	2.4±0.1	8.55±0.08
	(2.8)	(1.9)	(2.7)	(3.7)	(9.7)	(1.8)	(1.6)	(2.8)	(2.7)	(10.5)
S_{15}	217.5±2.08	0	274.3±22.9	41.2±0.7	112.8±1.2	107.7±0.93	0	362.3±26.2	44.5±0.8	67.9±0.06
	(64.4)	(0.0)	(94.4)	(78.5)	(86.8)	(57.8)	(0.0)	(97.0)	(78.5)	(83.0)
Σ	(99.2)	(91.4)	(97.1)	(104.2)	(106.3)	(100.1)	(99.8)	(99.8)	(100.1)	(108.4)

Proteins and chlorophyll content are given in μg per 10^6 living protoplasts, activity of phosphoenolpyruvate carboxylase (PEPC) and glucose-6-phosphate dehydrogenase (G6P DH) in μmol per sec per 10^6 living protoplasts, activity of ribonucleases (RNases) in U per 10^6 living protoplasts. S_0, P_1, P_{15} and S_{15} mean the crude homogenate, 1 000 and 15 000 *g* pellets and 15 000 *g* supernatant. Percentage values are shown in brackets.

increased markedly during the experimental period and both curves were correlated with the multiplication curve of the PVY^N (RNases: $r = 0.956^{***}$ for $n = 35$; G6P DH: $r = 0.974^{***}$ for $n = 38$). In contrast, no significant differences were found in the resistant line NCTG 83 (Fig. 1, Table 1).

Changes in the subcellular localisation of RNases and G6P DH isozymes were determined in mesophyll protoplasts prepared from healthy and PVY^N infected leaves of both the cultivars on day eight post inoculation (the culmination of multiplication curve of PVY and enhanced level of both enzymes in cv. Samsun). The pellet 1 000 *g* (P_1) of healthy protoplasts from cv. Samsun contained 93.5 % of chloroplasts (using the chlorophyll content as marker), 7.0 % of the total content of RNases and 15.1 % of G6P DH. The pellet from infected protoplasts (5.53 μg PVY^N per 10^6 living protoplasts, 57.8 ± 9.3 % of protoplasts was infected) contained 89.5 % of chloroplasts, 9.8 % of the total activity of RNases and 22.0 % of the G6P DH. The fraction of chloroplasts from infected protoplasts showed strongly enhanced content of RNases (192.4 % of healthy protoplasts, $P < 0.001$), and of G6P DH (174.4 %, $P < 0.001$). Cytosol (15 000 *g* supernatant, S_{15}) from infected protoplasts contained slightly enhanced activities of G6P DH (117.4 %) and considerably enhanced activities of RNases (141.7 %, $P < 0.001$) (Tables 2, 3). Activities of the mitochondria RNases were not analysed as no changes were observed in our previous experiments with PVY infected cv. Samsun (Šindelářová and Šindelář 1994). In the resistant line NCTG 83, significant differences were not observed in the activities and/or subcellular localisation of RNases as well as G6P DH isozymes. This is in accordance with no detectable content of PVY.

Table 3. Protein and chlorophyll content and phosphoenolpyruvate carboxylase (PEPC), glucose-6-phosphate dehydrogenase (G6P DH) and ribonucleases (RNases) activities in mesophyll protoplasts from susceptible (cv. Samsun) and resistant (transgenic breeding line NCTG 83) tobacco leaves inoculated with potato virus Y (necrotic strain). The results are expressed as percentages of this value found in the healthy protoplasts (S_0, P_1, P_{15} and S_{15} mean the crude homogenate, 1 000 and 15 000 *g* pellets and 15 000 *g* supernatant).

Cultivar	Fractions	Proteins	Chlorophyll	PEPC	G6P DH	RNases
Samsun	S_0	99.4	76.5 **	76.5 **	119.7 *	137.0 **
	P_1	87.4 *	73.2 **	-	174.4 ***	192.4 ***
	P_{15}	96.0	73.3 **	97.9	110.0	118.9
	S_{15}	101.6	-	76.9 *	117.4	141.7 ***
NCTG 83	S_0	97.9	81.9	98.5	103.0	90.6
	P_1	100.4	82.8	-	111.2	89.3
	P_{15}	96.8	86.3	98.9	99.8	93.8
	S_{15}	98.7	-	96.4	100.1	92.6

The results revealed a close connection between the metabolic pathways needed for PVY-RNA synthesis and the content of PVY. In cv. Samsun, enhanced activities of RNases and/or of G6P DH correlated with multiplication of PVY. The enhancement of RNases was a result of their increase both in chloroplasts and in

cytosol. The G6P DH was increased preferentially in chloroplasts. In the resistant breeding line NCTG 83 where no reproduction of PVY was observed, no changes in the activities of enzymes were found.

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