

## SHORT COMMUNICATION

**Regulation of Metabolic Pathways PVY-RNA Biosynthesis in Tobacco: Glycolytic Pathway**

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Tobacco plants infected with potato virus Y were studied at the stage of acute infection. Key enzymes of the glycolytic pathway, their regulation and the content of involved intermediates were monitored. The activities of the key enzymes of the glycolytic pathway (6-phosphofructokinase, fructosebisphosphatase and pyruvate kinase), determined in both crude homogenates and after partial purification, did not differ from the activities found in healthy control tissues. In virus-infected tissues the content of ATP was higher than in the healthy control. The levels of ADP and AMP decreased soon after inoculation, but increased at the end of the experimental period. The content of inorganic phosphate was not influenced by infection. No difference in adenylate energy charge was observed between healthy and virus-infected tissues. This implies that the rates of the glycolytic pathway *in vivo* are not altered during the acute stage of infection.

**Keywords:** ATP; ADP; AMP; fructosebisphosphatase; *Nicotiana tabacum* L. cv. Samsun; 6-phosphofructokinase; pyruvate kinase

In an infected host cell, virus RNA can be synthesised from intermediates of the reductive pentosephosphate pathway during photosynthesis and from intermediates of the oxidative pentosephosphate pathway active preferentially in the dark, or from intermediates released from degraded host rRNA. However, participation of these metabolic pathways in virus RNA biosynthesis usually depends on the type of virus and host, and on environmental conditions (ŠINDELÁŘOVÁ *et al.* 1997).

The resources for the activity of the glycolytic pathway and oxidative pentosephosphate pathway are free and transport carbohydrates, which are modified and phosphorylated by a complex

of invertases (saccharases) and hexokinases. The glycolytic pathway is regulated by ATP, ADP, and AMP at the level of hexokinases, 6-phosphofructokinase, fructosebisphosphatase and pyruvate kinase (TURNER & TURNER 1980).

Whereas the levels of activity of enzymes of the oxidative pentosephosphate pathway, invertases and hexokinases are increased in virus-infected tissues at the acute stage of infection, no change or only mild enhancement in the activities of enzymes of the glycolytic pathway were found (SOLYMOŠY & FARKAS 1963; HUTH 1973; MAKOVCOVÁ & ŠINDELÁŘ 1981; ŠINDELÁŘ 1986). In contrast, substantial changes in the rate of the glycolytic pathway, re-

lated presumably to the change in energy ratio, were observed at the chronic stage of infection (LADYGINA *et al.* 1966; ESANU 1969; MAKOVCOVÁ *et al.* 1980). Further, TÉCSI *et al.* (1994) observed that local infection with cucumber mosaic virus of cotyledons of *Cucurbita pepo* had a large stimulatory effect on the capacity for glycolysis at a later stage of infection.

This paper presents a study of regulative mechanisms of pathways of carbohydrate utilisation *via* glycolysis found in tobacco leaf tissues upon potato virus Y infection.

## MATERIAL 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  $\mu\text{mol}/\text{m}^2$  per s (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 & RICHTER 1978) at a concentration of 100  $\mu\text{g}/\text{cm}^3$ . 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).

Two systemically infected leaves of 10 plants were collected on the designated day and directly homogenised.

**Preparation of homogenate.** Homogenates (“homogenate”) were prepared from the samples by grinding in a mortar with fine silica sand, 10% (m/m) insoluble polyvinylpyrrolidone and TEMM buffer (20mM Tris/HCl buffer, 1mM EDTA, 2.5mM  $\text{MgCl}_2$ , 0.5mM PMSF, 1mM benzamidine, 1mM  $\epsilon$ -aminocaproic acid, 30mM 2-mercaptoethanol, pH 7.0) in a ratio of 1:5 (w/v). The resulting homogenate was squeezed through Miracloth and a nylon sieve 100 mesh and centrifuged for 10 min at 20 000 g.

The partially purified enzyme preparation (“purificate”) was obtained from 3  $\text{cm}^3$  of the crude homogenate by means of 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  $\text{cm}^3$  of TEMM buffer, desalted by centrifugation method through Sephadex G-25 Fine and filled up by buffer to the original volume of 3  $\text{cm}^3$  to enable comparison of the partially purified preparation

with the crude homogenate on a volume basis. Fractions below 0.2 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.

## Determination of enzyme activities

**Pyruvate kinase – PK (EC 2.7.1.40):** The assay mixture (1  $\text{cm}^3$ ) contained 100  $\mu\text{mol}$  TEMM buffer (pH 7.0), 100  $\mu\text{mol}$  KCl, 1  $\mu\text{mol}$  phosphoenolpyruvate, 2  $\mu\text{mol}$  ADP, 0.2  $\mu\text{mol}$  NADH, 10 U lactate dehydrogenase and 0.1  $\text{cm}^3$  of homogenate (NAKAYAMA *et al.* 1976).

**Phosphofructokinase – PFK (EC 2.7.1.11):** The assay mixture (1  $\text{cm}^3$ ) contained 100  $\mu\text{mol}$  TEMM buffer (pH 8.0), 3  $\mu\text{mol}$  ATP, 0.2  $\mu\text{mol}$  NADH, 4  $\mu\text{mol}$  F6P, 1 U aldolase, 10 U triosephosphate isomerase and 1 U glycerol-3-phosphate dehydrogenase and 0.1  $\text{cm}^3$  homogenate (PLAXTON 1990).

**Fructosebiphosphatase – FBP (EC 3.1.3.11):** The assay mixture (1  $\text{cm}^3$ ) contained 100  $\mu\text{mol}$  TEMM buffer (pH 7.5), 20  $\mu\text{mol}$  fructose-1,6-bisphosphate, 5  $\mu\text{mol}$  NADP<sup>+</sup>, 2 U PGI, 2 U G6P DH and 0.1  $\text{cm}^3$  homogenate (WOODROW *et al.* 1982).

Enzyme activities were determined at their respective pH optima at 25°C.

## Determination of Pi, ATP, ADP, AMP and AEC

**Inorganic phosphate (Pi) content** was determined according to COLE and ROSS (1966) after extraction with 0.25M of trichloroacetic acid.

**ATP, ADP and AMP contents** were determined spectrophotometrically after their extraction with 0.6M  $\text{HClO}_4$ . After neutralisation of the extract, the ATP content was determined enzymatically by glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase (ADAM 1963a), the ADP content by lactate dehydrogenase, and AMP content by pyruvate kinase and adenylate kinase (ADAM 1963b).

**The adenylate energy charge (AEC)** was determined by the equation according to ATKINSON and WALTON (1967):

$$\text{AEC} = [\text{ATP} + \frac{1}{2} \text{ADP}] / [\text{ATP} + \text{ADP} + \text{AMP}]$$

**Determination of PVY contents.** The PVY content was determined by quantitative DAS-ELISA

(CLARK & ADAMS 1977) with rabbit anti-PVY antibodies and alkaline phosphatase labelled 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).

**Statistical treatment and chemicals.** The results are presented as arithmetical means ( $\pm$  standard deviation of mean SDM) of three to five 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).

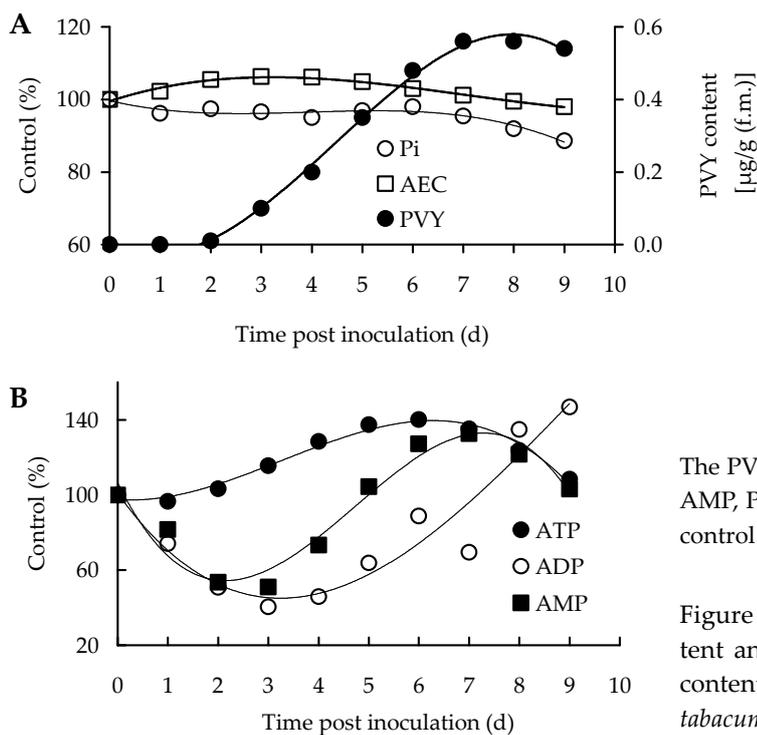
## RESULTS AND DISCUSSION

The PVY multiplication curve is shown in Figure 1A together with inorganic phosphate content (Pi) and AEC value. The Pi content ranges from 4.7 to 5.9  $\mu\text{mol/g}$  (f.m.) in healthy tissue. There was no significant difference in Pi content between infected and uninfected tissue as evaluated by the paired *t*-test ( $t = 1.172$  for  $n = 42$ ). Inorganic phosphate is a metabolic effector that takes part in regulation of glycolysis. In agreement with the findings of SASAKI and HIRAI (1963) and REBOWSKA (1971), we

did not find changes in Pi content in PVY-infected tissues (Figure 1A). This implies that inorganic phosphate does not have a regulatory effect on enzymes of the glycolytic pathway at the acute stage of virus-infection process.

The activities of key enzymes of the glycolytic pathway, 6-phosphofructokinase (PFK), fructose-bisphosphatase (FBPase) and pyruvate kinase (PK), were not influenced by PVY infection (data not shown; the statistical evaluation of paired *t*-test did not show any statistically significant difference between the healthy control and infected tissues; PFK:  $t = 1.280$  for  $n = 32$ ; FBPase:  $t = 1.062$  for  $n = 30$ ; PK:  $t = 1.134$  for  $n = 30$ ). The activities determined in both "homogenates" and "purificates" ranged from 9.97 to 14.15 (PFK), from 98.6 to 139.4 (FBPase) and from 188.4 to 244.4 (PK) nmol/g (f.m.) per min in "homogenates". In "purificates", the activities varied from 7.68 to 13.29 (PFK), from 98.9 to 128.1 (FBPase) and from 170.4 to 239.6 (PK) nmol/g (f.m.) per min. The enzyme activities in "homogenate" did not significantly differ from the corresponding purified enzyme preparation ("purificate") as evaluated by the paired *t*-test (PFK:  $t = 1.114$  for  $n = 38$ ; FBPase:  $t = 1.127$  for  $n = 36$ ; PK:  $t = 1.122$  for  $n = 36$ ).

The ATP, ADP and AMP content ranged from 87.5 to 98.1 (ATP), from 17.3 to 22.5 (ADP), and from 2.13 to 7.4 (AMP) nmol/g (f.m.) in healthy



The PVY content is given in  $\mu\text{g/g}$  (f.m.), the ATP, ADP, AMP, Pi and AEC value are expressed in % of healthy control

Figure 1. (A) The PVY, inorganic phosphate (Pi) content and AEC value; (B) The ATP, ADP and AMP content in systemically infected leaves of *Nicotiana tabacum* L. cv. Samsun

leaf tissues. The content of ATP (Figure 1B) was higher in PVY-infected leaf tissues than in those of the healthy control. The ADP and AMP (Figure 1B) contents decreased soon after inoculation, but increased at the end of the experimental period.

ATP, ADP, and AMP belong to a class of effectors which influence the glycolytic pathway at the level of hexokinases, 6-phosphofructokinase, fructosebisphosphatase and pyruvate kinase (TURNER & TURNER 1980). Regulation of these enzymes is a complex process in which, together with the nucleotides, the phosphoenolpyruvate, citrate, Pi, 6-phosphogluconate, 3- and 2-phosphoglycerate and presumably a number of other metabolites participate in the regulation at the level of metabolic intermediates (KELY & TURNER 1970). Although these enzymes have not yet been studied in detail in plants, it seems likely that 6-phosphofructokinase is inhibited by a higher content of ATP, ADP, AMP, phosphoenolpyruvate, 6-phosphogluconate and citrate; conversely, it may be stimulated by Pi and Mg<sup>2+</sup>. Pyruvate kinase is also inhibited by ATP and citrate, but enhanced by ADP (TURNER & TURNER 1980; PLAXTON 1996). Regulation of the glycolytic pathway by the content of purine nucleotides in virus-infected plants has not yet been studied; but some authors estimated the content of the nucleotides in virus-infected plants. BOZARTH and BROWNING (1970) monitored the content of purine and pyrimidine nucleotides in *Phaseolus vulgaris* infected with the southern bean mosaic virus and found a slightly increased content of endogenous ATP after 5 d. The content of other nucleotides remained unchanged and was not influenced by light-dark mode. SUNDERLAND and MERRETT (1967) and ŠINDELÁŘ (1986) reported a correlation between virus content and the content of ATP. KOZŁOWSKA (1967) found that an elevated level of ATP enhanced virus multiplication. A similar relationship between ATP content and PVY was found in this paper (Figure 1B).

Adenine nucleotides play a key role in the energy balance of cell and cell organelles. Energy balance and ratios in potato plants infected with PVY were studied by BOSER (1959) and by ESANU and SAVULESCU (1967), and by ESANU (1969) in tobacco plants infected with tobacco mosaic virus, who found a decrease in P/O quotient characterising oxidative phosphorylation of the mitochondria. LADYGINA *et al.* (1966) studied the energy balance and ratios in tobacco plants infected with tobacco mosaic virus. No changes were found in resistant tobacco plants,

while inhibition of oxidative phosphorylation was observed in susceptible plants.

In our experiments, the value of the adenylate energy charge AEC varied from 0.847 to 0.891 in healthy and from 0.847 to 0.940 in infected tissue. The AEC found in PVY-infected leaf tissues did not significantly differ from the healthy control (Figure 1A) as evaluated by the paired *t*-test ( $t = 1.118$  for  $n = 28$ ). This indicates a well-balanced adenylate energy system during the acute period of PVY infection and agrees with unaltered activities of 6-phosphofructokinase and pyruvate kinase which, as typical U-enzymes, are regulated by the AEC (TURNER & TURNER 1980). The results imply an unchanged rate of the glycolytic pathway on the base of a steady energy balance in the acute stage of PVY infection. This contrasts with conditions during the chronic stage of infection, according to findings by BOSER (1959), LADYGINA *et al.* (1966) and ESANU (1969).

## CONCLUSION

The activities of the key enzymes of the glycolytic pathway (6-phosphofructokinase, fructosebisphosphatase and pyruvate kinase) in PVY infected leaf tissue determined both in crude homogenates and after partial purification did not differ from the values found in healthy control tissues. The content of ATP was increased in virus-infected tissues, whereas ADP and AMP contents decreased just after inoculation and increased at the end of the experimental period. The content of Pi was not influenced by the infection.

The results indicate that the activities of the enzymes are not fine regulated by ATP, ADP, AMP and Pi content. The unchanged adenylate energy charge (AEC) value in virus-infected tissues implies that no changes in the rate of the glycolytic pathway occurred *in vivo* at the acute period of infection.

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**Souhrn**

ŠINDELÁŘ L., ŠINDELÁŘOVÁ M. (2004): **Regulace metabolických cest biosyntézy PVY-RNA v rostlinách tabáku: glykolýza.** Plant Protect. Sci., 40: 101–106.

V rostlinách tabáku infikovaného Y virem bramboru byla v období akutní fáze virového onemocnění studována aktivita klíčových enzymů glykolýzy, jejich hrubá a jemná regulace a obsah korespondujících cukerných intermediátů. Obsah anorganického fosfátu Pi v pletivech zdravých a infikovaných rostlin byl téměř shodný. Obsah ATP byl ve virózních pletivech zvýšen, obsah ADP a AMP po inokulaci pletiv poklesl, ale na konci akutní fáze infekce byl vyšší než u rostlin zdravých. Aktivity kontrolních enzymů regulace intenzity glykolýzy (6-phosphofruktokinasy, fruktosabisfosfatasy a pyruvát kinasy) nebyly virovým onemocněním statisticky významně ovlivněny. Enzymy, stanovené v surovém homogenátu a v jeho částečném purifikátu (kde byly odstraněny nízkomolekulární efekty, které by se mohly účastnit jemné regulace těchto enzymů), se od sebe vzájemně statisticky významně nelišily jak u zdravých, tak i u infikovaných pletiv. To naznačuje, že intenzita glykolýzy je u virózních rostlin určována hrubou regulací (tj. obsahem) jejich klíčových enzymů a ne jemnou regulací studovanými efekty. Hodnota adenylátového energetického náboje (AEC) byla ve sledovaném období u virózních a zdravých rostlin téměř shodná, což vede k předpokladu, že ani za podmínek *in vivo* nedochází u virózních pletiv ke změnám v intenzitách této metabolické dráhy.

**Klíčová slova:** ATP; ADP; AMP; fruktosabisfosfatasa; *Nicotina tabacum* L. cv. Samsun; 6-fosfofruktokinasa; pyruvát kinasa

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