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TMV-RNA biosynthesis in the light-green and dark-green regions of tobacco leaves

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Abstract

Changes in the proteins, chlorophyll, virus content and activity of key enzymes of viral RNA biosynthesis were investigated in the light- and dark-green regions of tobacco leaves systemically infected with tobacco mosaic virus. The protein content was increased to 118 % in the dark-green islands in contrast to 60 % in the light-green regions when compared with the control healthy leaves. The comparative analysis of soluble proteins from healthy and light- or dark-green regions of leaves by means of SDS-PAGE revealed that the main soluble proteins are equal in pattern but differ in quantity. The contents of chlorophylls did not differ from healthy tissues in the dark-green islands but were considerably lower in the light-green regions. The content of virus in light-green tissues was about 10 times higher than in the dark-green islands. The activities of key enzymes of oxidative pentosephosphate cycle – glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase – did not differ from healthy tissues in the dark-green islands but were considerably higher in the light-green tissues when compared with healthy control. Similar relationships were observed for ribonuclease, phosphomonoesterase and phosphodiesterase activities. The biosynthesis of viral RNA in the dark-green islands is probably restricted by the steady (or reduced) activities of key enzymes of these metabolic pathways.

Additional key words: chlorophyll, glucose-6-phosphate dehydrogenase, *Nicotiana tabacum* L., phosphodiesterase, 6-phosphogluconate dehydrogenase, phosphomonoesterase, proteins, ribonuclease, tobacco mosaic virus.

Introduction

Visual symptom of viral infection is chlorosis of leaf tissues. Marked reduction in photosynthetic activity arises early after inoculation in association with reduced size and number of chloroplasts, reduced chlorophyll content and low efficiency of CO_2 fixation in the chloroplasts (Jensen 1968, Tu *et al.* 1968). In addition, other considerable alterations in metabolism are observed, which leads to the damage of whole plant (*e.g.* Goodman *et al.* 1967, Šindelářová *et al.* 1997, 2000, Šindelář and Šindelářová 2002a).

Some of viruses give rise discrete dark- and light-green regions (islands) on systemically infected leaves (Fu1ton 1951, Atkinson and Matthews 1967, Hanušová *et al.* 1990, Guo and Garcia 1997, Yelina *et al.* 2002; Fig. 1). Dark-green islands (DGIs) have been focused on morphological and cytological analysis, even before the nature of viruses was known (Allard 1914). DGIs occur when a mosaic virus systemically infects a plant and gives rise to symptoms that include chlorosis of the leaves surrounding discrete regions of dark-green tissue. Typically, DGIs encompass more than one cell layer and contain more cells that can be accounted for if a DGI was the product of a single cell´s division. Some authors assumed the connection with concentration and subcellular localisation of abscisic acid (Whenham and Fraser 1981, Whenham *et al.* 1985, 1986). Cells within DGIs are free of viral RNA and proteins and also demonstrate resistance to superinfection by the original and closely related viruses but are susceptible to infection by unrelated viruses (Fulton 1951, Atkinson and Matthews 1970, Dougherty *et al.* 1994, Guo and Garcia 1997, Pang *et al.* 2000). The lack of virus in DGIs was used in regeneration of virus-free tobacco plants (Murakishi and Carlson 1976, Hanušová *et al.* 1990). Recovered tissue has been shown to undergo posttranscriptional degradation of the viral and the

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Abbreviations: G6PDH - glucose-6-phosphate dehydrogenase; PDE - phosphodiesterase; 6PGDH - 6-phosphogluconate dehydrogenase; PME - phosphomonoesterase; RNases - ribonucleases; rRNA - ribosomal RNA; TMV - tobacco mosaic virus.
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transgene RNA (Dougherty *et al.* 1994, Guo and Garcia 1997).

On the base of the distinct difference in viral content in dark- and light-green regions we studied metabolic pathways leading to precursors of viral RNA biosynthesis in these regions.

Materials and methods

Plant cultivation and virus inoculation: Two-month-old tobacco plants (*Nicotiana tabacum* L. cv. Samsun) grown under constant conditions in soil at an irradiance of 60 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 tobacco mosaic virus (TMV, common strain) (Gooding and Hebert 1967) at a concentration of 100 μg cm^{-3}. Corresponding leaves of control plants were mock-inoculated with distilled water. The samples of leaves from mock-inoculated or infected plants were collected on the 40th day post inoculation and the light-green or dark-green regions cut out with scalpel. The day of inoculation was designated as day zero.

Preparation of homogenate: Homogenates were prepared from the samples of light-green or dark-green regions 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 phenylmethylsulfonylfluoride, 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.

Electrophoresis of proteins: SDS-polyacrylamide gel electrophoresis (10 % resolving gel) according to Laemmli as described in Hames and Rickwood (1990) was performed to analyse soluble proteins. The gels were silver stained. Software *ImageMaster*^{TM} *TotalLab* (*Amersham Pharmacia Biotech*, Newcastle, UK) was used for analysis of lines.

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 assay was a modified procedure of Cheo (1971) by Šindelářová *et al.* (2000). The enzyme unit (U) was defined as the amount needed to cause an increase of 1.0 unit of absorbance at 260 nm in 1 h.

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, resp. 6.0) according to Chersi *et al.* (1966).

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) activity was determined spectrophotometrically (*Helios* type, *Unicam*, Cambridge, UK) (Šindelář *et al.* 1999).

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

Determination of TMV content: TMV content was determined by the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-TMV antibodies and alkaline phosphatase labeled antibodies raised against our isolate of TMV (common strain).

Statistical analysis and chemicals: The results in tables are presented as arithmetical means (± standard deviation of mean) of 3 - 7 measurements in three independent experiments. The *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

Protein content (Table 1) was markedly decreased to 60 % in the light-green regions, in contrast to mild increase up to 118 % in dark-green islands when compared with healthy control. This was confirmed by the comparative analysis of soluble proteins from healthy and light- or dark-green regions of leaves by means of SDS-PAGE, which revealed that the main proteins are equal in pattern but differ in quantity (Fig. 2).

Chlorophyll content in dark-green islands is analogous to healthy control in contrast to considerable decrease to 49.8 % in light-green regions. Similar pattern displays the composition of chlorophyll *a* and *b*. Chlorophyll *a* is decreased to 51.1 % and chlorophyll *b* to 46.6 % in light-green regions when compared with healthy control. The ratio of chlorophylls *a/b* characterizing integrity of two photosystems is the same

Table 1. The content of proteins, chlorophylls [mg g^{-1}(f.m.)], TMV [ng mg^{-1}(proteins)], the activities of G6P DH, 6PG DH, PME, PDE [nmol mg^{-1}(proteins) min^{-1}] and RNases [U mg^{-1}(proteins)] in the healthy and TMV-infected (common strain) light-green regions and dark-green islands of *Nicotiana tabacum* L. cv. Samsun leaves. The results in parentheses are expressed in percent compared to healthy control. The TMV content is compared to light-green regions (TMV content of light-green regions is substitute to 100 %). Means ± SE, * - the difference is statistically significant at $0.01 \le P < 0.05$, ** - $P < 0.01$ and *** - $P < 0.001$.

	Healthy	Light-green regions	Dark-green islands
Proteins	5.17 ± 0.24	3.12 ± 0.15 (60.4)***	6.11 ± 0.26 (118.2)*
Chlorophyll total	0.99 ± 0.06	0.49 ± 0.02 (49.8)***	0.96 ± 0.05 (97.5)
Chl *a*	0.71 ± 0.04	0.36 ± 0.02 (51.1)***	0.69 ± 0.03 (97.0)
Chl *b*	0.28 ± 0.01	0.13 ± 0.01 (46.6)***	0.28 ± 0.01 (99.3)
a/*b*	2.61 ± 0.03	2.36 ± 0.02*	2.58 ± 0.02
G6PDH	0.83 ± 0.05	1.17 ± 0.07 (141.3)***	0.84 ± 0.06 (100.8)
6PGDH	1.94 ± 0.15	2.48 ± 0.22 (128.1)**	1.82 ± 0.14 (93.9)
RNases	87.22 ± 6.42	143.52 ± 9.12 (164.6)***	82.21 ± 5.93 (94.0)
PME	56.28 ± 4.11	68.21 ± 5.31 (121.2)**	51.19 ± 3.79 (91.2)
PDE	10.13 ± 1.23	11.77 ± 1.42 (116.5)*	9.53 ± 1.08 (94.1)
TMV	-	412.34 ± 9.96 (100.0)	43.59 ± 0.92 (10.6)***

for healthy control and dark-green regions (2.61 and 2.58, respectively) compared to lower ratio (2.36) found in light-green regions. It suggests the suppression of photosynthetic rate (as well as the reductive pentosephosphate pathway) and in consequence the reduced availability of intermediates of photosynthesis for biosynthesis of viral RNA (Table 1).

Fig. 1. The dark-green islands and light-green regions on *Nicotiana tabacum* L. cv. Samsun leaves infected with tobacco mosaic virus (common strain) on the 40th day post inoculation.

Second source of intermediates for biosynthesis of viral RNA is oxidative pentosephosphate pathway, which degrades free and storage saccharides to ribose-5-phosphate needed to *de novo* biosynthesis of purine and pyrimidine nucleotides as precursors for synthesis of viral RNA. The control key of the pathway is complex G6PDH/6PGDH (Turner and Turner 1980, Šindelář and Šindelářová 2002b). Activities of G6PDH and 6PGDH in dark-green islands did not differ from healthy control (100.8 % and 93.9 %, respectively). In contrast, the activities of G6PDH and 6PGDH in light-green regions were significantly increased up to 141.3 and 128 %, respectively, when compared with healthy control. It indicates that the intensity of oxidative pentosephosphate pathway was increased, thus providing sufficient supply for intensive biosynthesis of viral RNA. On contrary, the intensity of this pathway in dark-green islands is similar

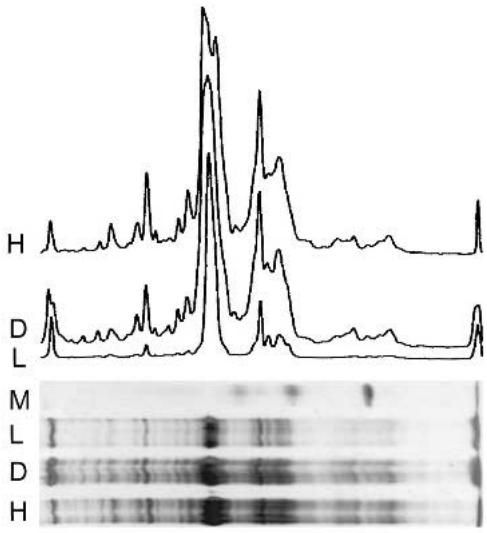

Fig. 2. SDS-PAGE analysis of soluble proteins from healthy (H), dark-green islands (D) and light-green regions (L) of *Nicotiana tabacum* L. cv. Samsun leaves infected with tobacco mosaic virus. The marker line (M) contains the markers of 45, 36, 29 and 14.2 kDa (from left to right).

to healthy tissues, which corresponds with low virus content (Table 1). The ratio G6PDH/6PGDH found in the healthy (0.43) and TMV-infected leaf tissues (0.47 for light-green, 0.46 for dark-green), which is lower than 1, confirms the assumption that G6PDH is the control enzyme of oxidative pentosephosphate pathway not only in the healthy but also in the light- and dark-green islands of TMV-infected plants.

Third source of intermediates for biosynthesis of viral RNA is degradation of host rRNAs, which could be characterised by the activities of RNases, PME and PDE. The activities of RNases, PME and PDE, similarly to enzymes above, are significantly increased in light-green regions (164.6 %, 121.2 %, resp. 116.5 %) opposite to slight decrease in dark-green islands (91 - 94 %) when compared with healthy control (Table 1). The increased activities of these enzymes likewise positively correlated with high content of virus in light-green regions.

Content of TMV (Table 1) in light-green regions estimated on the base of ELISA method is 10 times higher than that found in dark-green islands in accordance with determination of Atkinson and Matthews (1970) and Hanušová *et al.* (1990). Metabolic changes connected with virus biosynthesis in these types of tissues are still unknown.

In previous paper, Hanušová *et al.* (1990) observed more decisive contrasts when using ´alke´ strain of TMV, which induced more severe symptoms. In infected light-green regions of tobacco leaves, more than 250 times higher TMV content was found when compared with dark-green islands. Degradation of free and storage saccharides by oxidative pentosephosphate pathway (G6PDH) as well as the degradation of rRNAs (RNases) nearly doubled in light-green regions compared to healthy control. In contrast, the intensity of these pathways in dark-green islands was similar to healthy control and decreased in light-green regions. These results suggest lack of intermediates needed for TMV multiplication. The reduction or lack of TMV synthesis in dark-green islands was confirmed by regeneration of 28 % of healthy plants from callus cultures derived from dark-green islands. In addition, substantially decreased TMV multiplication was observed in other regenerated plants.

Conclusion: The content of virus in light-green regions of tobacco leaves infected with TMV (common strain) was about 10 times higher than in the dark-green islands. This probably results from the fact that degradation of free and storage saccharides by oxidative pentose-phosphate pathway as well as rRNAs by host's RNases, PME and PDE is considerably increased in light-green tissues thus supplying sufficient intermediates for viral biosynthesis. In contrast, any increase was not observed in dark-green islands, where the steady (or reduced) activities of key enzymes of these metabolic pathways are not sufficient for biosynthesis of intermediates of viral RNA.

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