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Dark and light green tissues of tobacco leaves systemically infected with tobacco mosaic virus

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Abstract

There are significant changes in the structure of the upper tobacco (*Nicotiana tabacum* L.) leaves systemically infected with tobacco mosaic virus (TMV) especially in the light green tissue (LGT). Dark green areas (DGI) had intermediate status between healthy tissue and LGT. DGI contained significantly less infectious TMV and viral antigen than the LGT. The DGI, LGT and healthy tissues did not differ in the permeability of cell membranes and in the set of acidic pathogenesis-related (PR) proteins but the total content of PR-proteins in the healthy plants was higher than in the infected ones with the DGI being intermediate between healthy tissue and LGT. The crude leaf extracts from DGI and LGT showed less total ribonuclease activity and ribonuclease isozymes in comparison with control.

Additional key words: Nicotiana tabacum, pathogenesis-related proteins, permeability of cell membranes, ribonuclease.

Introduction

In a sensitive host, virus spreads from primary infected cells over the whole plant, multiplies considerably and causes characteristic mosaic symptoms in the top leaves that include dark green areas, so-called dark green islands (DGI), surrounded by yellow or light green tissues (LGT). It was shown (Caldwell 1934, Fulton 1951, Solberg and Bald 1962, Atkinson and Matthews 1967, 1970, Loebenstein *et al.* 1977, Šindelářová and Šindelář 2004) that DGI contained little or no virus for a long period, though they were adjacent to and connected by plasmodesmata to heavily infected cells of LGT. Virus-free plants have been regenerated from protoplasts obtained from DGI (Shepard 1975, Murakishi and Carlson 1976). Furthermore, DGI were resistant to reinfection with the same or other strains of challenging virus causing mosaic symptoms but not to infection with local-lesion-forming strain of this virus or other viruses (Fulton 1951, Atkinson and Matthews 1970, Loebenstein *et al.* 1977). However, in the tobacco leaves systemically infected with tobacco mosaic virus (TMV) the number of DGI cells containing TMV particles as well as the number of TMV particles in them were increased when these leaves were infected with potato virus X (Reunov and Nagorskaya 1994).

Little is known about the development and biochemical basis for such resistance of DGI. The occurrence of DGI appears to be related to the age at which a leaf becomes invaded with virus (Solberg and Bald 1962, Reid and Matthews 1966, Atkinson and Matthews 1967, Takahashi 1971). From estimations of the numbers of cells present both in the leaf during leaf expansion and in the DGI, Atkinson and Matthews (1970) concluded that the DGI of the first leaf showing mosaic symptoms arose from a cluster of several hundred cells. It was shown (Atkinson and Matthews 1970, Honda and Matsui 1974) that DGI cells were cytologically similar to healthy leaves whereas LGT cells were abnormal. The cells of LGT were smaller than those in healthy leaves or DGI, and the cell arrangement was disordered. Degeneration of grana and lamellae, and occurrence of myelin-like structures in the chloroplasts were the most prominent modifications. However, Reunov (1999) found abnormal chloroplasts also in the cells of DGI.

Atkinson and Matthews (1970) proposed that the resistance of DGI was caused by some diffusible "dark

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Abbreviations: DGI - dark green area(s); d.p.i. - days post inoculation; LGT - light green tissue; LPI - leaf plastochron index; PR proteins - pathogenesis-related proteins; RNAse - ribonuclease; TMV - tobacco mosaic virus.
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green agent". Indeed, virus inhibitors were obtained from DGI (Wetzler and Schuster 1983, Gera and Loebenstein, 1988). An antiviral factor was isolated from extracts of TMV infected tobacco leaves. It restricted the virus infectivity *in vivo* and significantly decreased the activity of key enzymes of metabolic pathways tending to the nucleotides biosynthesis of viral RNA (Šindelářová and Šindelář 2003). It is unclear why these inhibitors have accumulated and functioned only in the cells of DGI but not in the neighboring cells of LGT. The protein content was increased up to 118 % in the DGI in comparison with healthy leaves, whereas in the LGT it came only to 60 % (Šindelářová and Šindelář 2004). It is possible that the resistance of DGI is associated with synthesis of pathogenesis-related (PR) proteins. PR-proteins are defined as plant proteins induced in response to the different stresses including attack by pathogens (Van Loon 1985). The nomenclature of PR-proteins consisting of 14 sets (PR-1 to PR-14) was created for all plants on the basis of amino acid sequences, serological, enzymatic and biological properties. Some of the PR-proteins are chitinases, 1,3-β-D-glucanases, ribonucleases, proteases and inhibitors of proteases (Van Loon and Van Strien 1999). By their properties, the PR-proteins can protect plants against the insect-pests, fungi and bacteria. To date, the role of PR-proteins in virus-resistance is not clear. So, ribonucleases (RNAses) seem to participate in plant defense mechanisms against viruses. Exogenous application of RNAses was reported to inhibit the development of viral pathogens, and RNAses were activated in infected plants (Diener 1961, Park *et al*. 2004, Šindelář and Šindelářová 2005, Šindelářová and Šindelář 2004, Šindelářová *et al*. 2000, 2002, 2005,). The accumulation of barley stripe mosaic virus was significantly reduced in transgenic plants expressing a bacterial RNAse (Zhang *et al.* 2001). Transgenic tobacco plants expressing the extracellular pancreatic ribonuclease exhibited a significantly higher level of protection against the virus infection (Trifonova *et al.* 2007). Currently, antiviral mechanisms of RNAses remain to be established.

The aim of this study was to compare the quantitative indices of leaf structure, the permeability of cell membranes, the accumulation of viral antigen and acidic PR-proteins, RNAse activity in the leaves of healthy plants and of the DGI and LGT of tobacco leaves systemically infected with TMV.

Materials and methods

Plants and virus: Tobacco plants (*Nicotiana tabacum* L., a sensitive cv. Samsun), were grown in pots in the greenhouse. To minimize the variability, leaf age was determined by plastochron index (LPI; Erickson and Michelini 1957) and not chronologically. Plants having leaves of LPI 18 to 20 were used in the experiments. Purified preparations of TMV Japanese strain OM (Nozu and Okada 1968) were obtained by polyethyleneglycol 6000 precipitation followed by three cycles of differential centrifugation (Boedtker and Simmons 1958) from the sap of the diseased tobacco plants (cv. Samsun) with well-expressed mosaic symptoms 14 d after inoculation. The average yield of purified TMV was *ca*. 1 g kg^{-1} of fresh leaf tissue.

The leaves with LPI from 10 to 15 were dusted with 400 mesh carborundum and inoculated by rubbing with TMV suspensions in 0.01 M phosphate buffer, pH 7.5 (100 μg cm^{-3}). Similar leaves of control plants were sham-inoculated with the same buffer. After inoculation the plants were cultivated at a 16-h photoperiod with irradiance of 57 μmol m^{-2} s^{-1} and temperature of 25 ± 1 °C.

Leaf structure analysis: Upper leaves with LPI from 3 to 5 were used in the following experiments. Discs (25 per sample, diameter 2 mm) were cut out from the DGI and LGT located in the middle part of leaf blades in 21st day post inoculation (d.p.i.) and fixed in 70 % ethanol. To determine the number of palisade and spongeous cells per unit of leaf area, the fixed discs were macerated by short heating in 50 % KOH at 80 - 90 °C. The cells were counted in a Goryaev hemocytometer under a light microscope. The number of chloroplasts per cell was determined following the maceration of discs in 5 % CrO_3 in 1 M HCL at 50 - 60 °C. Leaf thickness and cell linear dimensions were measured on transverse sections under a light microscope using an ocular micrometer. The volume of palisade cells was calculated using the equation (Goryshina 1989): $V = \pi(d/2)^2 (L-d + 2d/3)$, where d is cell diameter and L is cell length.

Permeability of cell membranes was determined by the electrolyte leakage method. Discs (50 per sample, diameter 2 mm) were cut out from the leaves with LPI from 3 to 5 in 21 d.p.i. and thoroughly rinsed with redistilled water to remove the matter from wounded edges. The samples were immersed in 10 cm^3 of redistilled water and incubated for 1 h with irradiance of 57 μmol m^{-2} s^{-1} and temperature of 25 ± 1 °C. Then the discs were discarded and the conductivity of water was measured using the conductometer *OK-102/1* (*Radelkis*, Budapest, Hungary) with electrode *OK-902*.

Virus assay: Discs (5 per sample, diameter 2 mm) were cut out from top leaves with LPI from 3 to 7 in 28 d.p.i. The viral antigen content in extracts of samples was determined by the method of Sapotsky *et al.* (2005). The sample infectivity was determined as described earlier (Malinovsky *et al.* 2001). The frozen samples were homogenized in 0.1 M phosphate buffer, pH 7.0 (1:50; m/v), containing 400 mesh carborundum. The homogenates were rubbed into the *Xanthi nc* leaves (a hypersensitive host of TMV). Each sample was inoculated into

15 - 20 leaf halves. The local lesions were counted in 3 d after inoculation.

Extraction and fractionation of acidic PR-proteins: Acidic PR-proteins were extracted by the method of Gianinazzi *et al.* (1977) in 28 d.p.i. and fractioned as described earlier (Sapotsky *et al*. 2005).

Ribonuclease assay: The RNase activity in crude leaf extracts of healthy plants and of DGI and LGT of leaves systemically infected with TMV was evaluated by the change in the amount of acid-soluble matter in total yeast RNA (Blank and McKeon 1989). Leaf tissue (1 g) was ground in liquid nitrogen, suspended in 1 cm^3 of 50 mM Tris-HCl (pH 7.5), and centrifuged for 300 s (8000 *g*, 4 °C). The extracts (0.02 cm^3) were added to the reaction mixture containing 4 % total yeast RNA, 1 % bovine serum albumin, 1 M Tris-HCl, pH 7.5, redistilled H_2O; the total volume was 0.06 cm^3. The samples were incubated for 1 h at 37 °C. Similar samples incubated for only 5 min were used as control. The reaction was stopped by adding 0.2 cm^3 of 3.4 % $HClO_4$. The test tubes were cooled to 4 °C and centrifuged at 8 000 *g* for 5 min. Redistilled H_2O (0.04 cm^3) was added to supernatants. The absorbance was measured at 260 nm relative to the control. One enzyme unit was defined as the amount of the enzyme causing a degradation of 1 µg RNA cm^{-3} s^{-1}.

Polyacrylamide gels were prepared under RNAse-free conditions according to the method of Yen and Green (1991) with minor modifications. Electrophoresis of the protein preparations (0.015 cm^3), containing *Ficoll* (5 %), sodium dodecyl sulphate (1 %), bromophenol blue and xylene cyanole F.F., was performed under sodium dodecyl sulphate-denaturing, non-reducing conditions using 1 mm thick 15 % polyacrylamide separating gel containing 2 mg cm^{-3} total RNA from baker's yeast, 0.2 mg cm^{-3} bovine fibrinogen, and 5 % stacking gel. The polyacrylamide gels were pre-run and the buffer changed before sample loading. After protein separation, the gels were washed twice with 0.01 M Tris-HCl buffer (pH 7.5) containing ethanol (70 %) to remove sodium dodecyl sulphate, and three times with 0.01 M Tris-HCl buffer (pH 7.5) to remove the ethanol, followed by incubation in 0.1 M Tris-HCl buffer (pH 7.5) for at least 1 h at room temperature. The gels were washed with 0.01 M Tris-HCl buffer (pH 7.5) and stained with 0.2 % toluidine blue O in the incubation buffer for 10 min (Yen and Green 1991), destained three times in 0.01 M Tris-HCl buffer (pH 7.5). The gels were scanned with the scanner *ScanPrisa 640S* (*Acer Inc*., Taoyuan, Taiwan; resolution of 2400).

Statistical treatment and chemicals: All experiments were performed in five replicates (five plants represented one replicate). The data of six independent experiments were combined, and those being above or below the arithmetical mean ± 2.96 (SD) were rejected as doubtful. The results in the tables and the figures are presented as arithmetical mean values with standard errors (SE). The *t*-test was employed to evaluate the statistical significance of differences.

Sephadex G-25 was obtained from *Pharmacia* (Uppsala, Sweden) and all other biochemicals were purchased from *Sigma* (St. Luis, MO, USA).

Results and discussion

The detection of the quantitative characteristics of leaf mesophyll structure is essential to understand the organization of plant photosynthetic apparatus and the adaptation of plants to environmental conditions. No significant differences between the leaves of healthy plants (control) and LGT of leaves systemically infected with TMV were found in the leaf thickness, the thickness of upper and lower epidermes, the number and length of abaxial stomata, the number and diameter of mesophyll palisade cells, and the number of chloroplasts in the palisade and spongeous mesophyll cells (Table 1). At the same time, the LGT contained more spongeous mesophyll cells per cm^2 of leaf surface and higher thickness of spongeous mesophyll than control. However, the thickness of palisade mesophyll, the length and volume of palisade mesophyll cells, and the content of chloroplasts in the palisade and spongeous cells were less for LGT in comparison with the healthy leaves.

Despite the fact that DGI looks visually like tissue of healthy leaves, viral infection affected DGI cells. Unlike the LGT, the DGI had a significantly greater leaf thickness, the thickness of upper and lower epidermes, the number of abaxial stomata. The palisade mesophyll cells of DGI were longer in comparison with those of the LGT (Table 1). Consequently, the leaf mesophyll structure of DGI was intermediate between healthy and LGT. In our opinion, the different influence of viral infection on the development of leaf tissues can be caused by the heterogeneity of leaves on the plant and even of tissues inside leaves (Avery 1933, Maxymowych 1963). Secondly, viral virions do not get simultaneously into all parts of plant and all plant cells.

The permeability of cell membranes increased during local lesion formation on leaves of hypersensitive plants infected with viruses (Ohashi and Shimomura 1976, Pennazio *et al*. 1979, Weststeijn 1978). Moreover, the permeability of cell membranes was higher not only in necrotic cells, but also in living cells bordering with local lesions (Seletskaya *et al.* 1996). In contrast, there were no changes of the permeability of cell membranes in the sensitive tobacco plants infected with TMV during 5 d.p.i. (Weststeijn 1978). We also found no differences in the permeability of cell membranes between the samples from DGI, LGT and similar leaves of healthy

Table 1. Quantitative characteristics of leaf structure and the permeability of cell membranes in healthy plants and light and dark green areas of tobacco leaves systemically infected with TMV. Means ± SE, n = 30, *, ** - differences between control and samples from diseased plants statistically significant at P = 0.05 and P = 0.01, respectively.

Characteristics	Healthy	DGI	LGT
Cell number of palisade mesophyll [$\times 10^3$ cm^{-2}]	340.0 ± 22.7	316.7 ± 14.3	290.5 ± 13.5
Cell number of spongeous mesophyll [$\times 10^3$ cm^{-2}]	121.8 ± 10.2	240.6 ± 17.8**	278.6 ± 18.3**
Chloroplast number per cell of palisade mesophyll	41.5 ± 1.9	33.5 ± 1.3*	31.8 ± 1.5*
Chloroplast number per cell of spongeous mesophyll	48.5 ± 1.8	33.9 ± 1.7**	35.3 ± 1.6**
Chloroplast number of palisade mesophyll [$\times 10^6$ cm^{-2}]	14.1 ± 1.4	10.6 ± 0.9	9.2 ± 0.9
Chloroplast number of spongeous mesophyll [$\times 10^6$ cm^{-2}]	5.9 ± 0.7	8.2 ± 1.0	9.8 ± 0.8
Length of palisade mesophyll cells [μm]	74.4 ± 1.2	70.6 ± 1.9	49.1 ± 1.4**
Diameter of palisade mesophyll cell [μm]	21.2 ± 0.8	16.7 ± 0.8*	18.9 ± 0.7
Volume of palisade mesophyll cell [$\times 10^3$ μm^3]	24.4 ± 1.8	15.4 ± 1.7*	12.2 ± 0.8**
The length of abaxial stoma [μm]	31.4 ± 1.2	29.5 ± 0.9	34.2 ± 0.6
Number of abaxial stomata [$\times 10^3$ cm^{-2}]	141.0 ± 3.0	179.0 ± 5.0**	143.0 ± 5.0
Leaf thickness [μm]	218.3 ± 3.3	249.0 ± 7.4**	221.8 ± 3.7
Thickness of palisade mesophyll [μm]	84.6 ± 2.8	74.4 ± 3.9	65.6 ± 1.9**
Thickness of spongeous mesophyll [μm]	131.3 ± 5.4	169.7 ± 9.1**	165.8 ± 4.6*
Thickness of upper epidermis [μm]	22.7 ± 1.7	27.7 ± 1.0	22.3 ± 0.7
Thickness of lower epidermis [μm]	17.4 ± 1.3	25.4 ± 1.3**	18.2 ± 1.1
Permeability of cell membranes [μS m^{-1}]	134.0 ± 13.0	122.0 ± 10.0	142.0 ± 11.0

plants in 21 d.p.i. (Table 1).

Viral antigen was found both in the DGI and LGT of the leaves with LPI from 3 to 7 in 28 d.p.i. The average content of viral antigen in DGI was significantly less in comparison with LGT (Figs. 1, 2). The accumulation of viral antigen was especially retarded in the DGI of the leaves having LPI from 3 to 6, however, the content of viral antigen was substantially raised in the DGI of the leaves with LPI 7 (characterized by increased dimensions and blurred boundaries of DGI). Similar results were obtained for infectious viral content in DGI and LGT of the leaves with LPI from 3 to 7. Contrary to reported data (Caldwell 1934, Fulton 1951, Solberg and Bald 1962, Atkinson and Matthews 1967, 1970, Loebenstein *et al.* 1977, Šindelářová and Šindelář 2004) we found that DGI contained quite a little amount of viral antigen and infectious virus, that can be caused by long time duration of disease (28 d.p.i. of lower leaves).

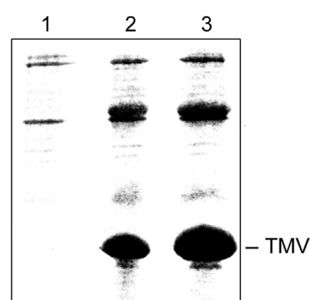

Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis profiles of viral antigen precipitated with specific antiserum from extracts of light and dark green tissues of leaves systemically infected with TMV in 28 d.p.i. *Lane 1* - healthy control, *lane 2* - dark green tissue, *lane 3* - light green tissue; TMV - band of viral antigen.

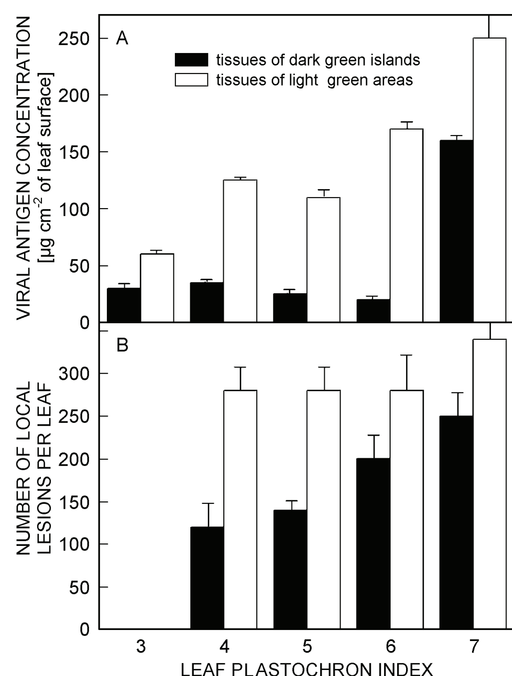

Fig. 2. The average content of viral antigen (*A*) and the sample infectivity (*B*) in the dark and light green areas of top tobacco leaves systemically infected with TMV.

There were no qualitative differences in acidic PR-proteins patterns between the leaves of healthy plants and of DGI and LGT of leaves systemically infected with TMV (Fig. 3), only quantitative differences were detected. The samples differed in the concentration of

various fractions of acidic PR-proteins. In total, the samples from LGT contained slightly less protein in the PR-bands than the samples from DGI and from healthy plants (Table 2). In contrast, the small accumulation of PR-proteins in sensitive plants infected with viruses was reported earlier (Van Loon and Van Kammen 1979, Fraser 1982, Profotova *et al.* 2007). The increase in the total content of PR-proteins in infected thorn-apple plants was observed during two weeks after infection but after three weeks post inoculation the differences were negligible (Sapotsky *et al.* 2005). Perhaps, a marginal decrease in the total content of PR-proteins in samples from diseased plants observed in our experiments can be explained by chronic stage of disease.

Table 2. The content of acidic PR-proteins [μg g^{-1} (f.m.)] in the extracts from healthy leaves and light and dark green tissues of tobacco leaves systemically infected with TMV. Means ± SE, n = 30, *, ** - differences between control and samples from diseased plants statistically significant at P = 0.05 and P = 0.01.

PR-bands	Healthy	DGI	LGT
P 1	9.69 ± 0.09	4.67 ± 0.45**	6.44 ± 0.38**
P 2	10.72 ± 0.34	5.15 ± 0.12**	7.74 ± 0.04**
P 3	2.23 ± 0.01	1.46 ± 0.06**	2.01 ± 0.08
P 4	3.21 ± 0.01	1.42 ± 0.01**	2.62 ± 0.16*
P 5	4.14 ± 0.18	3.30 ± 0,13*	3.58 ± 0.08
P 6	9.17 ± 0.95	9.67 ± 0.10	9.51 ± 0.58
P 7	10.05 ± 1.10	9.53 ± 0.25	10.97 ± 0.63
P 8	10.41 ± 0.97	9.07 ± 0.18	10.47 ± 0.37
P 9	12.27 ± 1.22	7.67 ± 0.56*	10.55 ± 0.38
P 10	22.93 ± 0.31	16.33 ± 0.56**	19.68 ± 0.25*
P 11	22.85 ± 0.25	24.29 ± 0.40	27.55 ± 0.52**
Total	117.67	92.56	111.12

Polyacrylamide gel electrophoresis of the extracts from leaves of healthy plants and DGI and LGT of tobacco leaves systemically infected with TMV displayed differences in the RNAses. All extracts contained two major proteins with RNAse activity and equal electrophoresis mobility. Leaf extracts of the healthy plants had also an additional minor band with RNAse activity (Fig. 4). Besides, four minor RNAse bands named as *a, b, c* and *d* were found in one experiment. The band *c* was observed in all extracts whereas the bands *a, b* and *d* were found in the extracts from LGT and healthy leaves, respectively (not shown). Many authors reported that RNAse activity was higher in diseased plants in acute stage of disease in comparison with healthy plants, but then it decreased to the control level or lower (for review, see Malinovsky 2002). Our experiments also showed that the crude leaf extracts from DGI and LGT showed lower total RNAse activity in comparison with control in 28 d.p.i. Total RNAse activities of crude leaf extracts from healthy leaves, DGI and LGT were 3.0 ± 0.05, 2.2 ± 0.15 and 2.5 ± 0.1 μg(degenerated RNA) cm^{-3} s^{-1}, respectively. However, in one experiment the extract from LGT had a significantly higher RNAse activity in comparison with other variants (not shown).

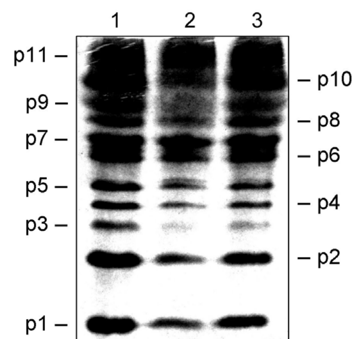

Fig. 3. Polyacrylamide gel electrophoresis profiles of acidic pathogenesis-related (PR) proteins from leaves of healthy tobacco plants and from light and dark green areas of leaves systemically infected with TMV in 28 d.p.i. *Lane 1* - healthy control, *lane 2* - light green tissue, *lane 3* - dark green tissue; p1-p11, bands of PR proteins.

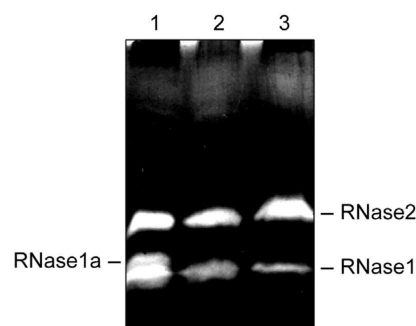

Fig. 4. Polyacrylamide gel electrophoresis profiles of proteins with ribonuclease activity from healthy tobacco plants and from light and dark green areas of leaves systemically infected with TMV in 28 d.p.i. *Lane 1* - healthy control, *lane 2* - dark green tissue, *lane 3* - light green tissue; RNAse1-2, major bands of RNAses; RNAse1a, minor band of RNAse.

It is unclear which feature of leaf cells might initiate DGI formation. According to data obtained, PR-proteins including RNAses do not take part in the resistance of DGI against viral infection. Perhaps, the cells of DGI have a higher ploidy in comparison with the cells of LGT. Chromosome numerical mosaicism is not a rare event (Rao and Nirmala 1986, Prakash *et al.* 1988). Higher ploidy in DGI gives rise to cell syntheses and thus decreases the chance of viral syntheses in competition for predecessor in developing cells and therefore, DGI contained greatly reduced amounts of viruses. It seems most probable that DGI are the result of posttranscriptional gene silencing (PTGS) (Moore *et al.* 2001). PTGS is known as a natural defense system against genetic stress factors, such as viruses (Ratcliff *et al.* 1997) and transposable elements (Ketting *et al.* 1999). PTGS is characterized by specific degradation of cytoplasmic RNAs and the accumulation of small 21- to

25-nucleotide fragments [small interfering RNAs (siRNAs)] of the targeted sequence (Waterhouse *et al.* 1999). It was shown that the siRNAs diagnostic of PTGS were accumulated in DGI (Yelina *et al.* 2002). A systemic PTGS signal moves both cell-to-cell and through the phloem (for review, see Mlotshwa *et al.* 2002). However, it is unclear why this signal operates only in the cells of DGI but not in neighboring cells of LGT. Moore and coauthors (2001) concluded that DGI and recovery were related phenomena but our results provide evidence that DGI have been infected with TMV during the development and ageing of leaves.

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