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Subcellular localization of ribonuclease isoenzymes in tobacco mesophyll protoplasts and their changes induced by infection of PVY

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Abstract

Changes in the content and subcellular localization of ribonuclease isoenzymes were determined in mesophyll protoplasts prepared from *Nicotiana tabacum* L. cv. Samsun from healthy and potato virus Y (PVY) infected plants. Intact chloroplasts, mitochondria and soluble cytosolic proteins were obtained after protoplast disintegration by means of differential centrifugation. The 1 000 *g* pellet from healthy protoplasts contained 7.3 %, the 15 000 *g* pellet 13.5 % and 15 000 *g* supernatant 82.1 % of the total activity of ribonucleases. The 1 000 *g* pellet from infected protoplasts contained 10.4 %, the 15 000 *g* pellet 10.0 % and 15 000 *g* supernatant 89.6 % of the total activity of ribonucleases. The activity of these enzymes in infected protoplasts was enhanced in crude homogenate to 137.0 % ($P < 0.001$), in 1 000 *g* pellet to 194.8 % ($P < 0.001$), in 15 000 *g* pellet to 101.3 % (NS), and in 15 000 *g* supernatant to 149.4 % ($P < 0.001$) of that in healthy noninoculated protoplasts.

Introduction

With respect to the degradation of ribonucleic acids in plant cells, information about the precise number of multiple forms, intracellular location and metabolic functions of the enzymes involved is fragmentary (Farkas 1982). Cytochemical and direct-isolation studies have revealed the vacuolar location of acid phosphatase, phosphodiesterase and nuclease activities (Matile 1978, Ryan and Walker-Simmons 1983). To obtain insight into the regulation of cellular RNA breakdown, extensive studies are needed to localize and purify the ribonucleolytic enzymes from the plant material.

The occurrence of RNA-degrading enzyme activity was demonstrated in extracellular space (Barna *et al.* 1989) but up to 80 % of RNA-splitting capacity was found in intracellular space. Boller and Kende (1979) and Abel and Glund (1986)

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indicated that the major vacuolar ribonucleolytic activity was identical with that of the major soluble plant endoribonuclease.

An increase in activity of ribonucleases (RNases) in plant tissues could be induced by wounding (Diener 1961, Bagi and Farkas 1967), by infection with fungi (Sutton and Shaw 1982, Barna *et al.*, 1989) or plant viruses (Reddi 1959, Diener 1961, Cheo 1971 *etc.*). RNases are not only typical stress enzymes, they play an important role in one of three ways of biosynthesis of virus-RNA from nucleotides released from degraded host rRNA (Reddi 1963, Cheo 1971, Šindelářová *et al.* 1988, Šindelář *et al.* 1990).

In spite of numerous studies of plant nucleases, little is known about the biological and pathological role and localization of these enzymes. The purpose of this study was to investigate subcellular localization of ribonuclease isoenzymes and changes induced by infection of potato virus Y, using the model of tobacco mesophyll protoplasts.

Materials and methods

Material: Tobacco (*Nicotiana tabacum* L., cv. Samsun) plants were grown in soil, at an irradiance of 60 μmol m^{-2} s^{-1} (photoperiod 12 h, *Philips HLRg* 400 W discharge lamps) and average temperatures of 25 °C.

Lower leaves, approximately 5 cm length (60-d-old plants), were mechanically inoculated with crude sap from leaves of plants infected with the potato virus Y (PVY, necrotic strain); corresponding leaves of control plants were treated with distilled water in the same way. The day of inoculation was designated as day zero.

Preparation and purification of protoplasts: Healthy or infected tobacco leaves (7 d after inoculation) were cut into 1 mm wide strips that were plasmolysed for 1 h at 25 °C in 10 mM MES-KOH buffer, pH = 5.5, which contained 0.45 M mannitol, 1 mM KNO_3,10 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM KH_2PO_4, 1 nM KI and 0.1 nM $CuSO_4$ (CPW medium, Cocking and Peberdy 1974). The medium was then replaced with the same one containing 1% (m/v) cellulase (*Onozuka R-10*) and 0.25 % macerase (*Macerozyme R-10*) and was vacuum infiltrated. To 6 g of strips, 60 cm^3 of this medium was applied. After 15 min at 30 °C the medium was centrifuged at 2000 *g* for 10 min and then replaced on the strips. Digestion of the cell walls was completed after 3 - 4 h at 30 °C. Cell-wall debris was removed by filtration through a 100 μm nylon sieve, and protoplasts were collected following centrifugation at 1500 *g* for 10 min. The sedimented protoplasts were resuspended in plasmolysing medium in a special 50 cm^3 conical tube, 45 cm^3 of 0.9 M sucrose (in plasmolysing medium) was placed beneath the protoplast suspension and centrifuged at 100 *g* for 2 min. Under these conditions, cellular debris sedimented and protoplasts floated to the surface. The protoplasts were removed by aspiration and diluted with 35 cm^3 of plasmolysing medium. Then 3 cm^3 of 10 mM MES-KOH buffer (pH 5.5), containing 0.6 M sucrose and 5 mM $CaCl_2$, was placed beneath the protoplast suspension and 3 cm^3 of 0.2 M $CaCl_2$ in 10 mM MES-KOH was layered above the protoplasts. The

tube was centrifuged at 100 *g* for 3 min, which resulted in two distinct bands in the gradient - an upper band of epidermal protoplasts and a lower, dark green band containing palisade parenchyma protoplasts (Fannin and Shaw 1987). The palisade parenchyma protoplasts were withdrawn with a Pasteur pipette, transferred to a 30 cm^3 tube and diluted to 20 cm^3 with 0.45 M mannitol. The protoplasts were washed twice with TEMMM medium (20 mM Tris-HCl buffer, 1 mM EDTA, 2.5 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 0.45 M mannitol, pH 7.0) centrifuged for 3 min at 100 *g* and resuspended in 9 cm^3 of TEMMM medium.

Breaking of protoplasts and fractionation of cell organelles: All steps of these procedures were carried out at a temperature 0 to 4 °C and monitored by light microscopy. Protoplasts were passed three times through a net with 20 μm meshes (*Pharmacia*, Uppsala, Sweden) which resulted in their complete disintegration. A 5 cm^3 aliquot of cell organelle suspension, designated as the crude homogenate S_0, was used for the determination of total protein and chlorophyll content, phosphoenolpyruvate carboxylase, cytochromoxidase and ribonuclease activities.

The remaining 4 cm^3 of cell organelles suspension were centrifuged for 10 min at 1 000 *g*. The P_1 pellet, containing the substantial part of the chloroplasts, was resuspended in 4 cm^3 of TEMM medium (TEMMM medium without the osmoticum). The supernatant was centrifuged for 15 min at 15 000 *g*. In this way, the supernatant S_{15} and the pellet P_{15} were obtained. The P_{15} pellet was resuspended in 4 cm^3 of TEMM medium.

Determination of number and viability of protoplasts, protein and chlorophyll content and enzyme activities: Protoplast number was determined in a haemocytometer and the number of viable protoplasts by staining with methylene blue according to Hooley and McCarthy (1980).

Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard, and chlorophyll content according to Arnon (1949).

The ribonucleases (RNases) assay was a modification of the procedure of Cheo (1971). Samples containing 0.1 cm^3 of homogenates were incubated with 0.3 cm^3 of yeast RNA (*Serva*, Heidelberg, Germany) (1.17 mg in 1 cm^3 of 0.15 M acetate buffer at pH 5.5, the optimum for RNases) for 60 min at 38 °C. After incubation the remaining RNA was precipitated with 3 cm^3 of precipitation solution for one hour at 0 °C. The mixture was centrifuged for 10 min at 5 000 *g* and the amount of degraded RNA in the supernatant was determined spectrophotometrically at 260 nm. The enzyme unit was defined as the amount of enzyme causing an increase in 260 nm absorbance of 1 000 units per hour. The activity of RNases assay was carried out under conditions of proportionality with respect to enzyme concentration and reaction time.

Phosphoenolpyruvate carboxylase activity (EC 4.1.1.31.) was determined according to Downton *et al.* (1971), cytochrome-c-oxidase activity (EC 1.9.3.1.) according to Simon (1958).

Enzyme activities were determined at 25 °C. The results presented are means of three to five determinations in four independent experiments.

PVY content assay: The PVY content in protoplasts was determined by the quantitative DAS-ELISA method (Clark and Adams 1977) with rabbit anti-PVY antibodies and alkaline phosphatase labelled antibodies prepared from our isolate of PVY necrotic strain.

Percentage of inoculated protoplasts assay: The samples of healthy and infected protoplasts were centrifuged from the incubation medium at 160 *g* for 10 min and the protoplasts were washed twice with 5 cm^3 cold 0.35 M KCl in 10 mM MES-KOH buffer pH 5.5, three times with 3 cm^3 cold 96 % ethanol and twice with PBS (0.15 M NaCl in 50 mM potassium phosphate buffer, pH 7.0), all for 2 min at 100 *g*. The fixed protoplast pellets were then resuspended in 1 cm^3 of PBS containing 2 mm^3 anti-TMV IgG conjugated to alkaline phosphatase and incubated for 16 h at 4 °C. After dilution of PBS, the samples were centrifuged for 5 min at 160 *g*. Most of the supernatant fluid was removed, the protoplasts were resuspended in 2 cm^3 TNM (0.1 M Tris-HCl, 0.1 M NaCl and 5 mM $MgCl_2$ buffer, pH 9.5) and centrifuged for 5 min at 160 *g*. The protoplast pellets were resuspended in 0.1 cm^3 TNM buffer and to them was added 2 - 5 mm^3 of a solution of BCIP and NBT (0.5 mg of 5-bromo-4-chloro-3-indolyl phosphate and 7.5 mg nitroblue tetrazolium diluted in 1 cm^3 of dimethyl formamide and then 2 cm^3 of TNM buffer added). The number of dark protoplasts and the total number of protoplasts per cm^3 were determined with a haemocytometer and the percentage of infected protoplasts in each sample was then calculated. Under these conditions less than 1.4 % of healthy protoplasts was stained.

Chemicals: Protoplast releasing enzymes were obtained from *Serva* (Heidelberg, Germany), alkaline phosphatase from *Boehringer* (Heisenhofen, Germany), and all other biochemicals from *Sigma Chemical Co.* (St. Louis, USA).

Results and discussion

Subcellular localization of ribonuclease isoenzymes in tobacco mesophyll protoplasts was determined by fractionation of cell organelles and of the cytosol, using the method of differential centrifugation. The advantages of utilizing the protoplast release technique when studying subcellular localization of enzymes are documented in Tables 1 and 2. They clearly show that intact chloroplasts (chlorophyll was used as a marker) were almost completely present in the 1 000 *g* pellet, and that *cca* 92 % of the cytosol (including vacuole contents), with the marker phosphoenolpyruvate carboxylase, were present in the 15 000 *g* supernatant. In healthy protoplasts about 82 % (82.1 ± 2.1) of ribonuclease activity was present in the 15000 *g* supernatant, which contained the cytosol plus vacuolar contents, about 7 % (7.3 ± 1.2) in the 1 000 *g* pellet and about 14 % (13.5 ± 1.8) in the 15 000 *g* pellet. In infected protoplasts (containing 4.21 μg PVY per 10^6 living protoplasts; 62.0 ± 4.6 % of protoplasts were inoculated) 90 % (89.6 ± 3.1) of ribonucleases were found in the 15 000 *g* supernatant, about 10 % (10.4 ± 1.9) in the 1 000 *g* pellet and 10 % (10.0 ± 2.1) in the 15 000 *g* pellet.

Table 1. Subcellular localization of ribonucleases in mesophyll protoplasts from *Nicotiana tabacum* L. cv. Samsun. Comparison of healthy plants and plants infected by potato virus Y (necrotic strain). Protein and chlorophyll contents are given in μg per 10^6 living protoplasts, activity of phosphoenolpyruvate carboxylase (PEPC) and cytochromoxidase (CYTOX) in nmol min^{-1} per 10^6 living protoplasts, activity of ribonucleases (RNases) in U per 10^6 living protoplasts. Percentage values are shown in brackets.

Fractions	Proteins	Chlorophyll	PEPC	CYTOX	RNases
Healthy plants					
Crude homogenate	395.0 (100.0)	90.0 (100.0)	26.41 (100.0)	13.20 (100.0)	110.4 (100.0)
1 000 *g* pellet	149.9 (38.0)	82.5 (91.7)	0.10 (0.5)	5.13 (38.9)	8.1 (7.3)
15 000 *g* pellet	13.7 (3.5)	1.9 (2.1)	0.62 (2.4)	8.76 (66.4)	14.9 (13.5)
15 000 *g* supernatant	252.1 (63.8)	0 (0)	24.14 (91.4)	0 (0)	90.6 (82.1)
Σ	(105.3)	(93.8)	(94.3)	(105.3)	(102.9)
Infected plants					
Crude homogenate	392.7 (100.0)	68.9 (100.0)	20.21 (100.0)	15.42 (100.0)	151.2 (100.0)
1 000 *g* pellet	129.8 (33.1)	60.3 (87.5)	0.11 (0.5)	6.25 (40.5)	15.7 (10.4)
15 000 *g* pellet	11.7 (3.0)	1.4 (2.0)	0.22 (1.1)	9.22 (59.8)	15.1 (10.0)
15 000 *g* supernatant	246.0 (62.6)	0 (0)	18.71 (92.6)	0 (0)	135.4 (89.6)
Σ	(98.7)	(89.5)	(94.2)	(100.3)	(110.0)

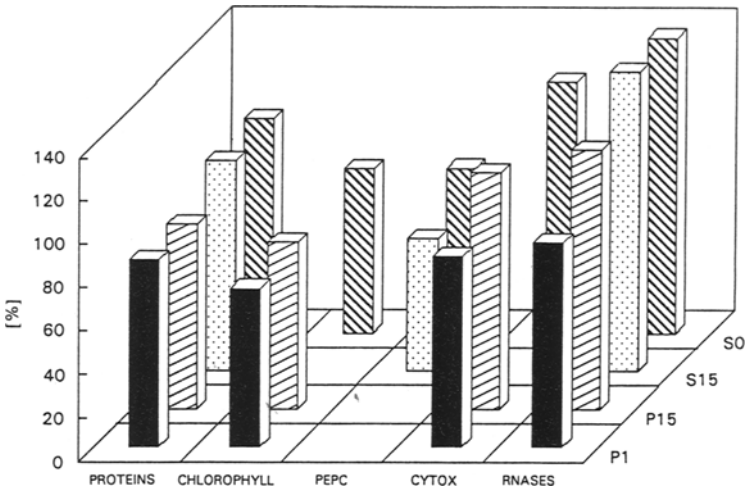

Fig.1. Distribution of proteins, chlorophyll, phosphoenolpyruvate carboxylase (PEPC), cytochromoxidase (CYTOX) and ribonucleases (RNases) in subcellular fractions prepared from tobacco mesophyll protoplasts infected with potato virus Y. The results are expressed as percentages of this value found in the healthy protoplasts (S_0 - crude homogenate, P_1 - 1 000 *g* pellet, P_{15} - 15 000 *g* pellet, S_{15} - 15 000 *g* supernatant).

In infected protoplasts the protein content in 1 000 *g* and 15 000 *g* pellets is lower (*ca.* 86 %), than in healthy protoplasts and the chlorophyll content is also lower in crude homogenate, 1 000 *g* and 15 000 *g* pellets (*ca.* 75 % of healthy protoplasts). The content of phosphoenolpyruvate carboxylase in infected protoplasts is lower in all fractions with the exception of the 1 000 *g* pellet. The content of ribonucleases in infected protoplasts is not changed in the 15 000 *g* pellet, but in the other fractions is strongly increased: in the crude homogenate up to 137.0 %, in the 1 000 *g* pellet up to 194.8 % and in the 15 000 *g* supernatant up to 149.4 % of healthy protoplasts (Fig. 1). The results demonstrate the fact that the virus infection enhances the activity of these isozymes both in chloroplasts and in cytoplasm.

The activities of complex of RNase isozymes in chloroplasts from infected protoplasts are doubled in comparison with healthy protoplasts. This corresponds with the findings of Oxelfelt (1971), Fraser (1972) and Suzuki and Taniguchi (1973) who described the strong degradation of chloroplast rRNA in infected tissue while cytoplasmic rRNA was degraded much less, mainly in the last phase of infection.

The role of chloroplasts in viral multiplication is not clear. Only a few plant viruses are multiplied directly in the chloroplasts or on their membranes (turnip mosaic virus, turnip yellow mosaic virus, tobacco etch virus - Matthews 1973, Gargouri and Haenni 1990). In consequence of viral infection, chloroplast rRNA is degraded and the activities of enzymes of the oxidative pentosephosphate cycle in chloroplasts are enhanced (Šindelářová and Šindelář, in press). From the results we conclude that in infected tissue of intact plants there were increased activities of these enzymes, mostly in consequence of increased contents of cytosolic (including vacuolar) and chloroplast isoenzymes.

References

Abel, S., Glund, K.: Localization of RNA-degrading enzyme activity within vacuoles of cultured tomato cells. - Physiol. Plant. **66**: 79-86, 1986.

Arnon, D.I.: Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-5, 1949.

Bagi, G., Farkas, G.L.: On the nature of increase in ribonuclease activity in mechanically damaged tobacco leaf tissues. - Phytochemistry **6**: 161-169, 1967.

Barna, B., Ibenthal, W.D., Heitefuss, R.: Extracellular RNase activity in healthy and rust infected wheat leaves. - Physiol. mol. Plant Pathol. **35**: 151-160, 1989.

Boller, T., Kende, H.: Hydrolytic enzymes in the central vacuole of plant cells. - Plant. Physiol. **63**: 1123-1132, 1979.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1977.

Cheo, P.C.: Effect in different plant species of continuous light and dark treatment on tobacco mosaic virus replicating capacity. - Virology **46**: 256-265, 1971.

Clark, M.F., Adams, A.N.: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. - J. gen. Virol. **34**: 473-483, 1977.

Cocking, E.C., Peberdy, J.F. (ed.): The Use of Protoplasts from Fungi and Higher Plants as Genetic Systems. A Practical Handbook. - University of Nottingham, Nottingham 1974.

Diener, T.O.: Virus infection and other factors affecting ribonuclease activity of plant leaves. - Virology **14**: 177-189, 1961.

Downton, L., Slatyer, R.O.: Variation in levels of some leaf enzymes. - Planta **96**: 1-12, 1971.

Fannin, F.F., Shaw, J.G.: Evidence for concurrent spread of tobacco mosaic virus from infected epidermal cells to neighboring epidermal and mesophyll cells. - Plant Sci. **51**: 305-310, 1987.

Farkas, G.L.: Ribonucleases and ribonucleic acid breakdown. - In: Parthier, B., Boulter, D. (ed.): Encyclopedia of Plant Physiology, N.S. Vol.14B: Nucleic Acid and Proteins in Plants II. Pp. 224-262. Springer-Verlag, Berlin - Heidelberg - New York 1982.

Fraser, R.S.S.: Effects of two strains of tobacco mosaic virus on growth and RNA content of tobacco leaves. - Virology **47**: 261-269, 1972.

Gargouri, R., Haenni, A.-L.: Replication of plant RNA viruses *in vivo* in relation to virus-host interactions. - In: Fraser, R.S.S. (ed.): Recognition and Response in Plant-Virus Interactions. NATO ASI Series, Vol. H41. Pp.163-183. Springer-Verlag, Berlin - Heidelberg 1990.

Hooley, R., McCarthy, D.: Extracts from virus infected hypersensitive tobacco leaves are detrimental to protoplast survival. - Physiol. Plant Pathol. **16**: 25-38, 1980.

Matile, P.: Biochemistry and function of vacuoles. - Annu. Rev. Plant Physiol. **29**: 193-213, 1978.

Matthews, R.E.F.: Induction of disease by viruses with special reference to turnip yellow mosaic virus. - Annu. Rev. Phytopathol. **11**: 147-170, 1973.

Oxelfelt, P.: Development of systemic tobacco mosaic virus infection. II. RNA metabolism in systemically infected leaves. - Phytopathol. Z. **71**: 247-256, 1971.

Reddi, K.K.: Studies on tobacco leaf ribonuclease, III. Its role in the synthesis of tobacco mosaic virus nucleic acid. - Biochim. biophys. Acta **33**: 164-169, 1959.

Reddi, K.K.: Studies on the formation of tobacco mosaic virus ribonucleic acid: II. Degradation of host ribonucleic acid following infection. - Proc. nat. Acad. Sci. USA. **50**: 75-81, 1963.

Ryan, C.A., Walker-Simmons, M.: Plant vacuoles. - Colowick, O.P., Kaplan, N.O. (ed.): Methods of Enzymology. Vol. 93. Pp. 580-589. Academic Press, New York - San Francisco - London 1983.

Simon, E.W.: The effect of digitonin on the cytochrome c oxidase activity of plant mitochondria. - Biochem. J. **69**: 67-74, 1958.

Šindelář, L., Šindelářová, M., Čeřovská, N., Hanušová, M.: Changes in ribonuclease and glucose-6-phosphate dehydrogenase activities during PVY-RNA biosynthesis in potato leaf discs. - Biol. Plant. **32**: 119-127, 1990.

Šindelářová, M., Šindelář, L., Hanušová, M.: [Changes in the activities of ribonuclease and glucose-6-phosphate dehydrogenase during the TMV-RNA biosynthesis in tobacco leaf discs.] - Ochr. Rost. (Praha) **24**: 87-93, 1988. [In Czech.]

Sutton, B.C.S., Shaw, M.: Changes in two ribonuclease isozymes during rust infection of flax cotyledons. - Plant Physiol. **69**: 205-209, 1982.

Suzuki, T., Taniguchi, Y.: Degradation of host high molecular weight RNA in barley leaves infected with barley stripe mosaic virus. - Phytopathol. Z. **77**: 55-64, 1973.