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Changes in composition of soluble intercellular proteins isolated from healthy and TMV-infected *Nicotiana tabacum* L. cv. Xanthi-nc

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Abstract

Changes in ribonucleases (RNases), phosphomonoesterase (PME), phosphodiesterase (PDE), glucose-6-phosphate dehydrogenase (G6P DH), polyphenoloxidases, peroxidases and proteases activity and PR-proteins composition in leaf tissue and intercellular fluid (ICF) isolated from leaf tissue of healthy and TMV-infected hypersensitive tobacco (*Nicotiana tabacum* L. cv. Xanthi-nc) plants (non-inoculated leaves) were studied. The amount of the proteins and the enzymes of intercellular space was less than 3 % of the total amount of proteins and the enzymes found in homogenate of healthy leaves. The TMV infection did not significantly change this observation. The great increase in the activities of the enzymes was observed in homogenates of the infected leaves, especially of the enzymes involved in biosynthesis of precursors needed for virus multiplication (G6P DH, RNase, PME, PDE). This is in contrast with the activities of the enzymes of ICF, which were only partly increased. The ICF proteins of infected plants were separated by means of ion exchange chromatography on DEAE cellulose. The isozymes of peroxidase, polyphenoloxidase, PME and PDE were identified. Using discontinuous nondenaturating polyacrylamide gel electrophoresis of DEAE cellulose fractions, the detection of isozymes of peroxidases and PR-proteins was performed. By means of SDS-PAGE the molecular masses of PR-proteins were identified: 15 - 16 kDa (group 1), 27 - 28 kDa (group 3: chitinases) and 36 - 40 kDa (group 2a: β-1,3-glucanases).

Additional key words: glucose-6-phosphate dehydrogenase, PAGE, peroxidases, phosphodiesterase, phosphomonoesterase, polyphenoloxidases, proteases, PR-proteins, ribonucleases, tobacco mosaic virus.

Introduction

Pathogenesis-related proteins (PR-proteins) are plant species-specific proteins produced in response to infection with viruses, fungi or bacteria. Their occurrence and some of their biochemical properties have been reviewed (Antoniw and White 1983, Redolfi 1983, Van Loon 1985). PR-proteins were first identified in tobacco reacting hypersensitively to tobacco mosaic virus (TMV), where they have been assumed to limit multiplication and/or spread of the invading virus (Van Loon and Van Kammen 1970). Four PR-proteins accumulating around the developing local necrotic lesions on the leaves (Antoniw and White 1986, Rohloff and Lerch 1977) have been relatively well characterized. These comprise components designated PR la, lb and lc, each consisting of a single polypeptide and having similar molecular mass (15 kD) and amino acid compositions (Antoniw *et al.* 1980), and PR 2, of higher molecular mass (Gianinazzi *et al.* 1977). These proteins are present in large amounts in infected leaves: PR la may constitute about 1 % of the soluble protein 7 d after infection (Antoniw and Pierpoint 1978). Currently, five families of PR proteins, numbered 1 - 5, have been classified (for review see Linthorst 1991). Within each family, members of the so-called class I are generally localized in the vacuole, whereas class II proteins occur extracellularly. Proteins of the PR-2 family have β-1,3-endoglucanase activity, and those of the PR-3 family have endochitinase activity, but plants expressing either gene alone are not

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Abbreviations: dpi - day post inoculation; G6P DH - glucose-6-phosphate dehydrogenase; ICF - intercellular fluid; PAGE - polyacrylamide gel electrophoresis; PDE - phosphodiesterase; PEROX - peroxidases; PME - phosphomonoesterase; PPO - polyphenol-oxidases; PROT - proteases; RNases - ribonucleases; SAR - systemic acquired resistance; TMV - tobacco mosaic virus.

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significantly protected. These results suggest that the accumulation of PR proteins is causally related to the onset of systemic acquired resistance (SAR). However, definite evidence for the role of PR proteins in SAR is still lacking.

However, a number of additional proteins (polyphenoloxidases, ribonucleases, peroxidases, proteases, *etc.*) have been reported in intercellular fluid of tobacco (Bol *et al.* 1990). This paper presents the results of a detailed study of changes in activities of ribonucleases, phosphomonoesterases, phosphodiesterases, polyphenoloxidases, peroxidases, and proteases, and PR proteins composition in ICF isolated from leaf tissue of hypersensitive tobacco cultivar Xanthi-nc induced by TMV infection.

Materials and methods

Plant cultivation and virus inoculation: Two-month-old tobacco plants (*Nicotiana tabacum* L. cv. Xanthi-nc) grown under constant conditions in Hoagland No. 3 nutrient solution, at an irradiance of 100 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 10 μg cm^{-3}. Corresponding leaves of control plants were mock-inoculated with distilled water. The samples of upper 20 non-inoculated leaves from 10 mock-inoculated or infected plants were collected in 7th day post inoculation (dpi) and used for ICF and crude tissue homogenate preparation. The day of inoculation was designated as day zero (0 dpi).

Protein extraction and ICF electrophoresis: Intercellular fluid (ICF) of leaves was collected by vacuum infiltration with the isolation medium (20 mM tris-HCl buffer pH 8.0, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM 2-mercaptoethanol and 0.5 M sucrose) followed by centrifugation (2 000 *g* for 10 min). The remaining tissue of leaves were homogenised 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 $MgCl_2$ and 30 mM 2-mercaptoethanol, pH 7.0) in a ratio 1:5 (m/v). The homogenate was squeezed through *Miracloth* and nylon sieve 100 mesh and centrifuged for 10 min at 20 000 *g*. Preparation and storage of the crude homogenates and ICF were carried out at 0 to 4 °C (activity of the enzymes did not change for more than 5 h).

Discontinuous nondenaturating polyacrylamide gel electrophoresis in 1 mm thick 10 % and 12.5 % resolving gel and 4 % stacking gel was performed to analyse acidic proteins and SDS-polyacrylamide gel electrophoresis (12.5 % resolving gel) according to Laemmli as described in Hames and Rickwood (1990).The gels were silver stained or assayed for peroxidase activity. After nondenaturating PAGE the gel was soaked for 15 min in 0.1 M acetic buffer pH 5.5 and then immersed for 30 min in the staining solution containing of 0.025 M guaiacol and 0.1 % H_2O_2 in 0.1 M acetic buffer pH 5 at 25 °C. The dark brown bands of peroxidases appear.

DEAE column chromatography: ICF was desalted by centrifugation method through *Sephadex G-25 Fine* and applied to a column of *DE-52* cellulose with 35 cm^3 bed volume previously equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 2.5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol). After washing with one bed volume of buffer A, the proteins were eluted with 60 cm^3 of a linear gradient of Tris-HCl between 20 and 100 mM, followed by 240 cm^3 of a linear gradient of KCl between 0 and 500 mM in buffer B (the same composition as buffer A, but with 100 mM Tris-HCl). Fractions of 6 cm^3 were collected, desalted by centrifugation passage through *Sephadex G-25 Fine* and assayed for proteins content and enzymes activity. All operations were performed at 0 - 4 °C.

Determination of protein content and enzyme activities: Protein content was determined according to Bradford (1976) using bovine serum albumin as a standard.

The ribonucleases (RNases) activity assay was a procedure of Cheo (1971) modified by Šindelářová *et al.* (2000a). 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 (G6P DH, EC 1.1.1.49) activity was determined spectrophotometrically (*Helios* type, *Unicam*, Cambridge, UK) (Šindelář *et al.* 1999), phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was also determined spectrophotometrically at 340 nm (Downton and Slatyer 1971). Polyphenoloxidases (PPO, EC 1.10.3.1) were determined according to Van Kammen and Brouwer (1964), peroxidases (PEROX, EC 1.11.1.7) according to Lück (1963) and complex of proteases (PROT) according to Lusso and Kuć (1995).

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 prepared against our isolate of TMV common strain.

Statistical treatment and chemicals: The results in tables are presented as arithmetical means (± standard deviation of mean) of 3 - 7 measurements in four 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

The PEPC and G6P DH of ICF were used as markers for the contamination of ICF by cellular proteins. The fact that only low activities of these enzymes were observed in ICF of healthy as well as the infected tobacco (PEPC 0.06 and 0.07 %, and G6P DH 0.34 and 0.24 %, respectively) demonstrates a high purity of ICF. No significant difference between the protein content of the healthy and the infected plants was observed in homogenates (Table 1). However, the increase in proteins to 126.5 % was found in ICF of leaves from infected plants, probably as a consequence of newly synthesized pathogenesis-related proteins (Table 2). The proteins of ICF contribute 2.68 % to total protein content of infected plants whereas the contribution of only 2.08 % was observed in healthy plants. Similarly, the activities of the enzymes of intercellular space did not exceed 3 % of the enzymes found in homogenate (Table 3).

A great increase in the activities of enzymes in homogenates of infected plants, especially of the enzymes involved in biosynthesis of the precursors needed for the synthesis of TMV-RNA (G6P DH, RNase,

Table 1. Protein content [mg g^{-1} (f.m.)], activities of glucose-6-phosphate dehydrogenase (G6P DH) and phosphoenolpyruvate carboxylase (PEPC) [nmol g^{-1} (f.m.) s^{-1}], ribonucleases (RNases of pH optima 5.5 and 7.0) [U g^{-1} (f.m.)], phosphomonoesterases (PME) and phosphodiesterases (PDE) [μmol g^{-1}(f.m.) s^{-1}], polyphenoloxidases (PPO), peroxidases (PEROX) and proteases (PROT) [ΔA g^{-1} (f.m.) s^{-1}] in crude tissue homogenate. * - the difference is statistically significant at $0.01 \le P < 0.05$; ** - at $0.001 \le P < 0.01$; and *** - at $P \le 0.001$.

	Healthy	Infected	[%]
Proteins	4.42 ± 0.12	4.32 ± 0.17	97.7
G6P DH	0.74 ± 0.02	1.25 ± 0.02	169.0***
PEPC	1.67 ± 0.03	1.57 ± 0.04	94.1
RNases pH 5.5	96.08 ± 1.21	136.84 ± 2.81	142.4***
RNases pH 7.0	23.42 ± 0.25	32.43 ± 0.74	138.5***
PME	44.72 ± 1.06	56.20 ± 1.33	125.7**
PDE	7.33 ± 0.08	11.33 ± 0.17	154.5***
PPO	ND	ND	-
PEROX	0.73 ± 0.02	1.19 ± 0.03	163.2***
PROT	0.006 ± 0.00	0.007 ± 0.00	116.7*

Table 2. Protein content [μg g^{-1} (f.m.)], activities of G6P DH and PEPC [pmol g^{-1} (f.m.) s^{-1}], RNases [U g^{-1} (f.m.)], PME and PDE [μmol g^{-1} (f.m.) s^{-1}], PPO, PEROX and PROT [ΔA g^{-1} (f.m.) s^{-1}] in intercellular fluid (abbreviations are described in Table 1). * - the difference is statistically significant at $0.01 \le P < 0.05$.

	Healthy	Infected	[%]
Proteins	92.08 ± 0.83	115.65 ± 1.04	125.6
G6P DH	2.50 ± 0.16	3.00 ± 0.12	120.0
PEPC	1.02 ± 0.02	1.17 ± 0.04	114.7
RNases pH 5.5	2.41 ± 0.01	3.00 ± 0.01	124.5*
RNases pH 7.0	0.60 ± 0.01	0.71 ± 0.01	118.3
PME	0.535 ± 0.035	0.656 ± 0.04	122.5
PDE	0.070 ± 0.004	0.087 ± 0.01	124.2*
PPO	0.003 ± 0.000	0.002 ± 0.00	93.3
PEROX	0.021 ± 0.001	0.026 ± 0.00	123.8*
PROT	0.0002 ± 0.000	0.0002 ± 0.00	100.2

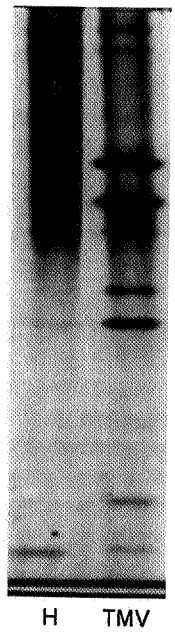

Fig. 1. Native PAGE of acidic proteins of intercellular fluid from healthy (H) and tobacco mosaic virus (TMV) infected tobacco.

Table 3. Protein content and activities of enzymes in crude homogenate and intercellular fluid (ICF) in per cent of ICF in homogenate (abbreviations are described in Table 1).

	Healthy	Infected
Proteins	2.08	2.68
G6P DH	0.34	0.24
PEPC	0.06	0.07
RNases pH 5.5	2.50	2.19
RNases pH 7.0	2.56	2.19
PME	1.19	1.17
PDE	0.95	0.77
PPO	-	-
PEROX	2.88	2.19
PROT	2.78	2.27

PME, PDE) was observed (Table 1). This corresponds to our previous findings (Šindelářová *et al.* 1997, 1998, 2000a,b, Šindelář *et al.* 1999). On contrary, the activities of enzymes of ICF are increased only slightly (118 - 125 %).

The infection of hypersensitive tobacco with TMV induced biosynthesis of PR-proteins (Fig. 1). The method of ion exchange chromatography on DEAE cellulose was used for separation of PR-proteins of intercellular fluid. The enzymes (or isozymes, respectively) of peroxidase, polyphenoloxidase, PME and PDE were separated and identified (Fig. 2). The activities of other enzymes were not high enough to be successfully identified, so they are not presented. The purity of the fractions was furthermore tested using the electrophoretic separation under native

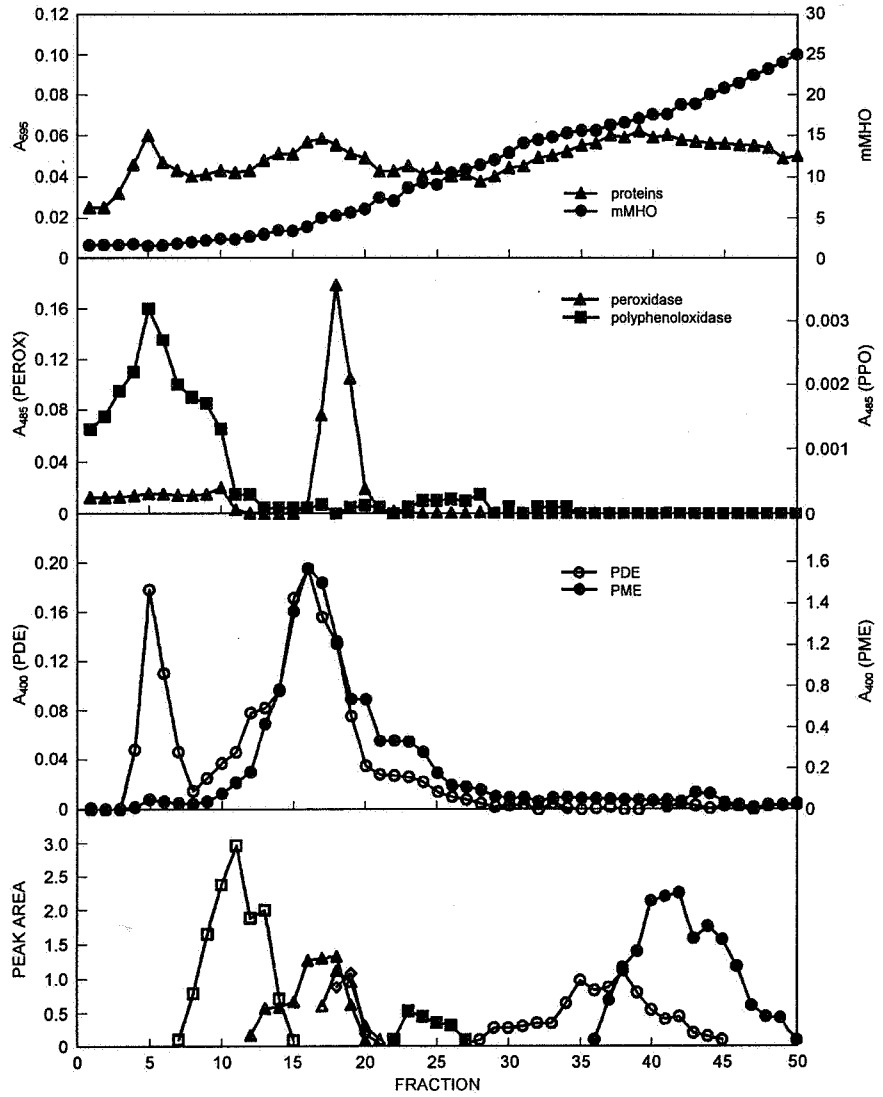

Fig. 2. Elution profile of intercellular fluid of upper non-infected leaves of TMV-inoculated hypersensitive tobacco obtained by using ion exchange chromatography on DEAE cellulose (*A*, *B*, *C*) and the peak areas (*D*) of the proteins of Rf 0.08 (*open squares*), 0.28 (*closed triangles*), 0.31 (*open triagles*), 0.33 (*rhombs*), 0.48 (*closed squares*), 0.56 (*open circles*) and 0.90 (*closed circles*) established by native PAGE of the individual fractions. PEROX - peroxidases, PPO - polyphenoloxidases, PDE - phospho-diesterases, PME - phosphomonoesterases.

condition (data not shown). Quantitative estimation of the particular proteins content is demonstrated in Fig. 2*D* with the electrophoretic relative factors (Rf) included. Isozymes of peroxidase were detected in fractions 16 - 22 (Fig. 2*B*). In other experiment where the gradient of Tris-HCl was omitted, the isozymes of peroxidase were better fractionated (Fig. 3).

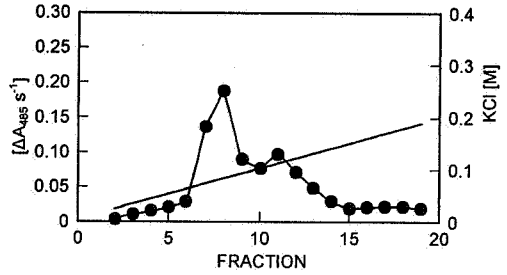

Fig. 3. Elution profile of the selected fractions (2 - 19) containing peroxidase activity obtained from DEAE cellulose column using elongated gradient of KCl.

The proteins of representative fractions 25, 35 and 47 were subdivided by SDS-PAGE (Fig. 4) and their molecular masses estimated. In all studied fractions, the proteins of 36 - 40 kDa were found, which correspond to PR-proteins of group 2a (β-1,3-glucanases) as referred in Bol *et al.* (1990). PR-proteins of 15 - 16 kDa (group 1) were detected in fractions 25 and 47. In fraction 25, the protein of 27 - 28 kDa possessing chitinase activity (detection of chitinase in gel is not shown) was determined (PR-proteins; group 3).

No virus was detected in the non-inoculated tobacco leaves as well as in ICF of infected plants. Consequently, the observed changes in enzymes activities and PR-proteins were caused by mechanisms of induced resistance and not by process involved in biosynthesis of TMV.

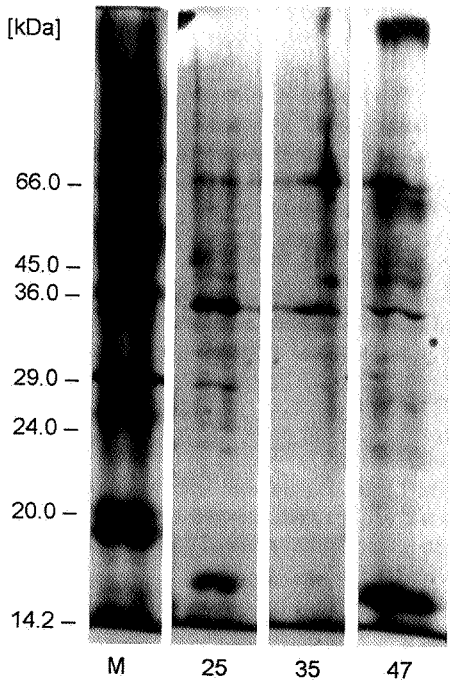

Fig. 4. SDS-PAGE proteins obtained from fraction 25, 35 and 47 of DEAE cellulose column.

Conclusion: The activities of enzymes, especially of those involved in biosynthesis of precursors needed for TMV-RNA (G6P DH, RNases, PME, PDE), were significantly increased in leaf homogenates of inoculated tobacco on contrary to the slightly increased activities of ICF. The ICF proteins of inoculated plants contained the isozymes of peroxidase, polyphenoloxidase, PME, PDE (separated by means of ion exchange chromatography on DEAE cellulose) and PR-proteins of the molecular mass 15 - 16 kDa (group 1), 27 - 28 kDa (group 3: chitinases) and 36 - 40 kDa (group 2a: β-1,3-glucanases).

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