Isolation of Pathogenesis-Related Proteins from TMV-Infected Tobacco and their Influence on Infectivity of TMV

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Abstract

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The composition of pathogenesis-related proteins (PR-proteins) in the intercellular fluid (ICF) and leaf tissue of the hypersensitive tobacco cultivar Xanthi-nc inoculated with *Tobacco mosaic virus* (TMV), and their inhibitory influence on TMV multiplication were studied. The ICF PR-proteins of infected plants were separated after solubilisation by decreasing gradient of ammonium sulphate, the cell PR-proteins were separated after acidic homogenisation of leaf tissues. The ICF and cell PR-proteins were further purified by ion exchange chromatography on DEAE cellulose. Using discontinuous non-denaturating polyacrylamide gel electrophoresis of DEAE cellulose fractions the PR-proteins were detected. Their molecular weights were estimated by SDS-PAGE. The ICF and cell proteins of infected leaves included PR-proteins of the molecular weights 15–16 kDa (Group 1), 27–28 kDa (Group 3: chitinases) and 36–40 kDa (Group 2a: β-1,3-glucanases). Fractions with different PR-proteins were tested for their effect on infectivity of TMV. Particularly the PR3 and PR2a proteins seem to decrease the infectivity of TMV.

Keywords: PR-proteins; PAGE; *Tobacco mosaic virus*

Abbreviations: TMV – *Tobacco mosaic virus*; ICF – intercellular fluid; SAR – systemic acquired resistance; PAGE – polyacrylamide gel electrophoresis; PR-proteins – "pathogenesis-related" proteins

Pathogenesis-related proteins (PR-proteins) are plant species-specific proteins induced specifically in pathological or related situations. They are not only accumulated locally in the infected leaf, but are also induced systemically, and are associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses. Induction of PR-proteins has been found in many plant species belonging to various families, suggestive of a general role for these proteins in adaptation to biotic stress conditions. Their occurrence and some of their biochemical properties have been reviewed (Antoniw & White 1983; Van Loon & Van Strien 1999). PR-proteins were first identified in tobacco reacting hypersensitively to TMV and were assumed to limit multiplication and/or spread of the invading virus (Van Loon & Van Kammen 1970). Originally, five main classes of PR-proteins, numbered 1–5, were characterised by both biochemical and molecular-biological techniques in tobacco (for a review see Linthorst 1991). Within each family

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of PR-proteins, members of the so-called class I are generally localised 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-3,4 families have endochitinase activity. Later, other families of PR-proteins were recognised. By then, 14 families were classified (Van Loon & Van Strien 1999).

In addition, a number of additional proteins (polyphenoloxidases, ribonucleases, peroxidases, proteases etc.) have been reported in intercellular fluid of tobacco (Bol *et al.* 1990; Šindelářová & Šindelář 2001).

This paper presents the results of a detailed study of the composition of PR-proteins in ICF and leaf tissues isolated from the hypersensitive tobacco cultivar Xanthi-nc induced by TMV infection, and their specific inhibitory effect on virus multiplication.

MATERIALS AND METHODS

Plant cultivation and virus inoculation. Two-month-old tobacco plants (*Nicotiana tabacum* L. cv. Xanthi-nc) grown under constant conditions in soil, at an irradiance of 100 μmol/m^2/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 *Tobacco mosaic virus* (TMV, common strain) (Gooding & Hebert 1967) at a concentration of 100 μg/cm^3. Samples of the upper 20 non-inoculated leaves from 10 mock-inoculated and 10 TMV inoculated plants were collected on the 7th day post inoculation and used for ICF and crude tissue acidic homogenate preparation. The day of inoculation was designated as day zero (0 day post inoculation = 0 dpi).

Protein extraction. Intercellular fluid (ICF) of leaves was collected by vacuum infiltration with the isolation medium IM (20mM tris-HCl buffer pH 8.0, 10mM $MgCl_2$, 10mM $CaCl_2$, and 10mM 2-mercaptoethanol) followed by centrifugation (2000 g for 10 min). The remaining tissue of leaves was homogenised by grinding in a mortar with fine silica sand, 10% (w/w) insoluble polyvinylpyrrolidone and PCN buffer (100mM phosphate/citrate buffer, 500mM NaCl and 0.1% 2-mercaptoethanol, pH 3.0) in a ratio 1:1.25 (w/v). The homogenate was squeesed through Miracloth and nylon sieve of 100 mesh and centrifuged for 10 min at 20 000 g. The supernatants, named "acidic homogenate" and "ICF", were precipitated to 90% saturation with ammonium sulphate. Preparation and storage of the acidic homogenates and ICF were carried out at 0–4°C.

Purification of ICF. The precipitate of ICF was centrifuged (10 min at 16 000 g), resuspended in 10 cm^3 IM and added stepwise to 20 cm^3 Sephadex G-25 Coarse equilibrated with 90% ammonium sulphate dissolved in IM. The chromatographic column was filled with 10 cm^3 equilibrated Sephadex, Sephadex including the sample of ICF and completed with equilibrated Sephadex to the final volume 50 cm^3. ICF proteins were eluted by ammonium sulphate gradient (from 90% to 0% saturation dissolved in IM) with the rate of 40 cm^3/h and fraction volumes 3 cm^3. The ammonium sulphate concentration of fractions was monitored on the base of conductivity measurement and content of proteins by spectrophotometric determination of absorbance (A 280 nm). The fractions containing the individual PR-proteins were combined, desalted and applied to a column of DE-52 cellulose. All operations were performed at 0–4°C.

DEAE column chromatography. The samples of ICF and acidic homogenate were desalted by means of the 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 (20mM tris-HCl pH 8.0, 1mM EDTA, 2.5mM $MgCl_2$ and 10mM 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 100mM, followed by 240 cm^3 of a linear gradient of KCl (from 0 to 500mM) in a buffer B (the same composition as buffer A, but with 100mM Tris-HCl). Fractions of 6 cm^3 were collected, desalted by centrifugation passage through Sephadex G-25 Fine and assayed for proteins content and antiviral activity. All operations were performed at 0–4°C.

Protein electrophoresis. Discontinuous non-denaturating polyacrylamide gel electrophoresis in 1 mm thick, 10% 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. Software ImageMaster^{TM} TotalLab (Amersham Pharmacia Biotech, Newcastle, UK) was used for analysis of lines.

Biological test for PR-proteins activity on TMV content. Purified TMV was prepared as described

by Gooding and Hebert (1967). A mixture of equal volumes of diluted TMV (10 µg TMV/cm^3) and PR-proteins was used for the inoculation of hypersensitively reacting *N. tabacum* L. cv. Xanthi-nc half-leaves (half-leaves method). A solution of TMV only (final concentration 5 µg TMV/cm^3) was applied as a control on the opposite half-leaf. The fractions separated by DEAE-cellulose chromatography were applied in the same way with exception that the solution of PR-proteins was replaced with desalinated fraction. The effectiveness of PR-proteins to reduce the infectivity of TMV was calculated by taking the number of lesions found on half-leaves treated with a mixture of TMV and PR-protein as percentage of the number of lesions on the control half-leaves inoculated only with TMV.

Statistical treatment and chemicals. The results in the tables are presented as arithmetical means (± standard deviation of mean) of three to seven measurements in four independent experiments. The *t*-test was employed to characterise the differences.

The biochemicals were purchased from Sigma Chemical Company (St. Louis, USA).

RESULTS AND DISCUSSION

The inoculation of the hypersensitive *Nicotiana tabacum* L. cv. Xanthi-nc with TMV induced biosynthesis of PR-proteins in both ICF and leaf tissue. The method of ion exchange chromatography on DEAE cellulose afforded partial separation of isolated PR-proteins. The purity of the fractions was furthermore analysed using electrophoretic separation under native condition and molecular weights were estimated by means of SDS-polyacrylamide gel electrophoresis with molecular weight standards.

PR-proteins of the ICF

The spectrum of PR-proteins of the ICF induced by TMV inoculation and that found in the mock-inoculated control is shown in Figure 1. Bands with low Rfs correspond to peroxidase and polyphenoloxidase activities; bands with higher Rf are PR-proteins (Šindelářová & Šindelář 2001).

Proteins of ICF induced by TMV inoculation were purified by precipitation with ammonium sulphate (AS) followed by gradual solubilisation with gradient of ammonium sulphate from 90 to 0% of saturation (Figure 2). Fractions 25–30 were combined and named sample AS I, fractions 31–33 named sample AS II, and 34–37 sample AS III.

The samples were separated and purified by ion-exchange chromatography on DEAE cellulose (Figure 3). After DEAE chromatography, specific fractions were combined: the fractions 21–28 of AS I (named DEAE I), fractions 18–26 of AS II (named DEAE II), and fractions 19–27 of AS III (named DEAE III). The spectrum of proteins in the combined fractions was tested by means of PAGE using a Laemmli non-dissociating buffer system (Figure 4), and molecular weights of proteins were estimated by analysis of SDS-PAGE with a molecular weight standard (data not shown). In sample DEAE I, a PR-protein (R_f = 0.58) of 27–28 kDa was determined (PR-protein – group 3 – chitinase activity), in sample DEAE II (R_f = 0.58 and 0.80), proteins of 27–28 kDa and 36–40 kDa were found, which correspond to PR-proteins of Group 3 and the PR-protein group 2a (β-1,3-glucanases), and PR-proteins of 15–16 kDa (Group 1) were detected in sample DEAE III (R_f = 0.93).

The effects of samples DEAE I, II and III on the infectivity of TMV were tested on cv. Xanthi-nc. Sample DEAE I with the PR3 protein (chitinase)

Table 1. Inhibitory effect of PR-proteins (PRs) isolated from ICF on the infectivity of TMV on *Nicotiana tabacum* L. cv. Xanthi-nc

Sample	Group of PRs	Number of lesions		TMV reduced by PRs (% of control)
		TMV only	TMV + PRs	
Crude ICF	all proteins	89.3 ± 13.1	41.7 ± 5.2	46.9***
DEAE I	PR3	116.7 ± 15.4	59.6 ± 6.4	51.1***
DEAE II	PR3 + PR2a	166.2 ± 21.1	71.1 ± 8.2	42.8***
DEAE III	PR1	117.9 ± 14.4	105.6 ± 9.7	89.6*

Means ± SE, *the difference is statistically significant at $0.01 \leq P < 0.05$ and ***$P < 0.001$

Figure 1. Non-denaturating PAGE of acidic proteins of ICF of healthy (H) and tobacco mosaic virus (TMV) inoculated plants of *Nicotiana tabacum* L. cv. Xanthi-nc

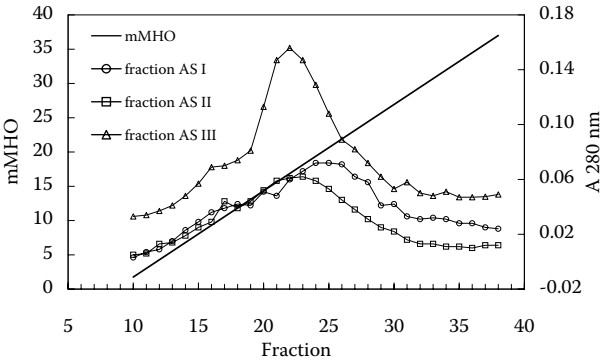

Figure 3. Elution profiles of combined fractions AS I (open circles), AS II (open squares) and AS III (open triangles) originated from ICF, separated by ion-exchange chromatography on DEAE cellulose

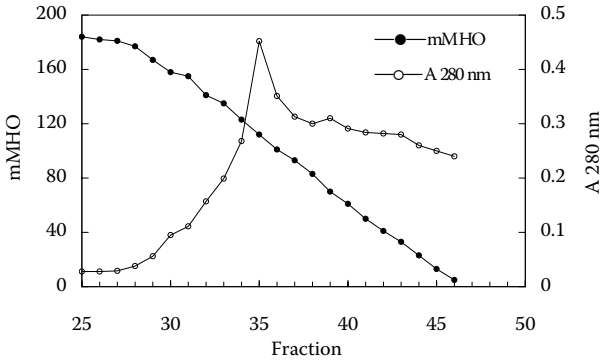

Figure 2. Elution profile of ICF isolated from leaf tissues of TMV-inoculated tobacco obtained by ammonium sulphate solubilisation

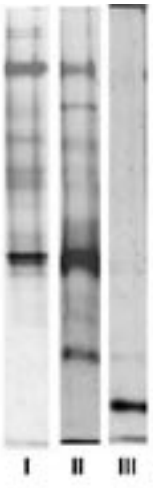

Figure 4. Non-denaturating PAGE of proteins originated from ICF in combined fractions DEAE I (I), DEAE II (II) and DEAE III (III) separated by ion-exchange chromatography on DEAE cellulose

decreased the infectivity of TMV to 51.1%, sample DEAE II with PR2a/PR3 proteins (β-1,3-glucanase and chitinase) to 42.8% and sample DEAE III with PR1 protein to 89.6% (Table 1).

PR-proteins of the acidic homogenate

A similar spectrum of proteins as in the ICF was found in the acidic homogenate prepared by acidic homogenisation of leaf tissue remaining after elimination of ICF. Proteins of the acidic homogenate were separated and purified by ion-exchange chromatography on DEAE cellulose (Figure 5). The DEAE fractions were analysed by means of non-denaturating PAGE (Figure 6). Fractions 15, 20 and 30 were selected on the base of different spectra of proteins and their molecular weights were estimated after SDS-PAGE (data not shown). A similar pattern of PR-proteins as for ICF was found. Fraction 15 contained predominantly the protein of 27–28 kDa (PR-proteins of Group 3), fractions 20 the proteins of 27–28 kDa and 36–40 kDa (PR-proteins of Group 3 and 2a), and fraction 30 the PR-proteins of 15–16 kDa (Group 1).

All DEAE fractions were tested for their effect on infectivity of TMV (Figure 7). Data from non-denaturating PAGE and test of infectivity imply

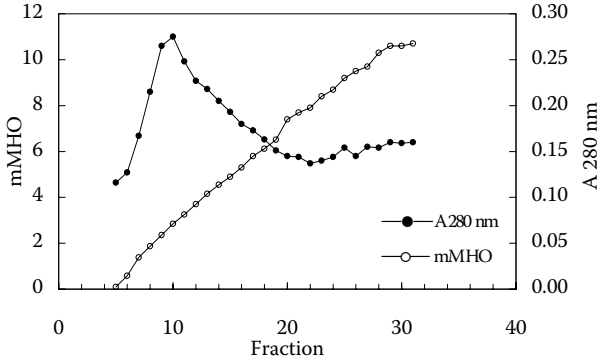

Figure 5. Elution profiles of acidic homogenate proteins separated by ion-exchange chromatography on DEAE cellulose

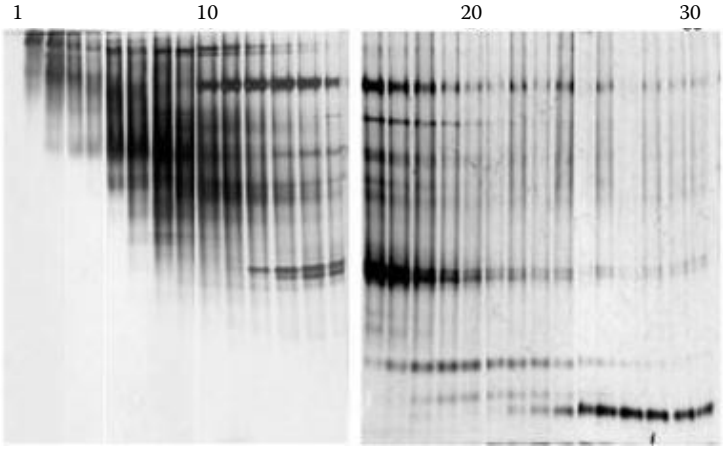

Figure 6. Non-denaturating PAGE of acidic homogenate proteins in fractions separated by ion-exchange chromatography on DEAE cellulose

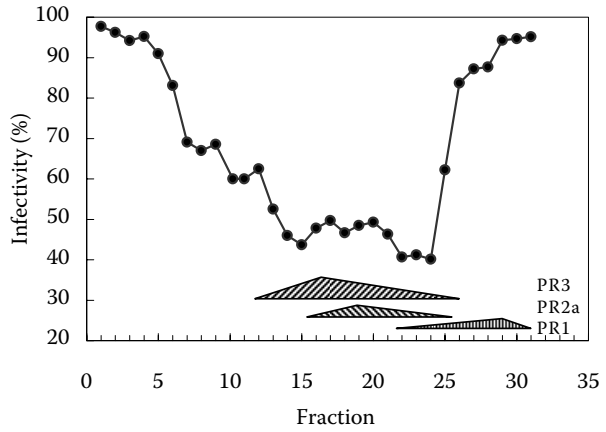

Figure 7. Effect on the infectivity of TMV by fractions of acidic homogenate proteins separated by ion exchange chromatography on DEAE cellulose. Upper triangle represents the content of PR3, intermediate triangle the content of PR2a and bottom triangle the content of PR1 in fractions

that mostly the PR2a and PR3 proteins participate in inhibition of TMV infectivity on *Nicotiana tabacum* L. cv. Xanthi-nc.

CONCLUSION

The ICF and acidic homogenate proteins of inoculated plants contained the same PR-proteins of the molecular weights 15–16 kDa (Group 1), 27–28 kDa (Group 3: chitinases) and 36–40 kDa (Group 2a: β-1,3-glucanases). Predominantly the PR3 and PR2a proteins seem to decrease the infectivity of TMV.

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Abstrakt

Šindelářová M., Šindelář L. (2005): **Izolace „pathogenesis-related" proteinů z tabáku infikovaného virem TMV a jejich vliv na infektivitu viru TMV**. Plant Protect. Sci., **41**: 52–57.

Z listů hypersenzitivního kultivaru tabáku Xanthi-nc infikovaného virem *Tobacco mosaic virus* (TMV) byly izolovány „pathogenesis-related" proteiny (PR-proteiny) jak z intercelulárních prostorů (ICF), tak ze zbývajících pletiv, a byl studován jejich vliv na multiplikaci TMV. PR-proteiny ICF byly purifikovány solubilizací klesajícím gradientem síranu amonného, buněčné proteiny kyselou homogenizací zbývajících listových pletiv. ICF a buněčné PR-proteiny byly dále purifikovány iontoměničovou chromatografií na DEAE celulóze. Jednotlivé PR-proteiny byly detekovány diskontinuální nedenaturující elektroforézou na polyakrylamidovém gelu (PAGE) a jejich molekulové hmotnosti byly určeny pomocí SDS-PAGE. Buněčné i ICF proteiny obsahovaly stejné PR-proteiny o molekulových hmotnostech 15–16 kDa (skupina 1), 27–28 kDa (skupina 3: chitinasy) a 36–40 kDa (skupina 2a: β-1,3-glukanasy). Infektivitu TMV významně inhibovaly PR-proteiny skupin PR3 a PR2a.

Klíčová slova: PR-proteiny; polyakrylamidový gel; *Tobacco mosaic virus*

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