

## Soluble pathogenesis-related proteins of *Capsicum annuum* leaves induced by cucumber mosaic virus infection

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### Abstract

Eight major pathogenesis related (PR) proteins were found in a soluble extract of cucumber mosaic virus (CMV) infected *Capsicum annuum* leaves. None of them was present in the soluble extract of sham-inoculated controls. The proteins were detected by two-dimensional polyacrylamide gel electrophoresis using (2D-PAGE) *Phast-System*, native-PAGE in the first dimension and SDS-PAGE in the second. Two major acidic PR-proteins were identified on the basis of their relative molecular mass ( $M_r$ ), PR1 of 15.3 kD and PR2 of 29 kD. Six proteins were basic and were identified as PR1a of 15.9 kD, PR1b of 15.0 kD, PR1c of 15.9 kD, PR2 of 27.0 kD, PR3 of 36.3 kD and PR4 of 48.8 kD.

### Introduction

In response to infection by various types of pathogens or to treatment with selected chemicals, several plant species react by accumulating a set of low-molecular-mass proteins called PR-proteins. Such proteins were first identified in tobacco (*Nicotiana tabacum*) cultivars reacting hypersensitively to tobacco mosaic virus (TMV) (Gianinazzi *et al.* 1970, Van Loon and Van Kammen 1970) and have since been detected in over 20 plant species. The occurrence of these new proteins is not

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**Abbreviations:** CMV - cucumber mosaic virus, 2D-PAGE - two-dimensional polyacrylamide gel electrophoresis,  $M_r$  - relative molecular mass, PAGE - polyacrylamide gel electrophoresis, PDT - *PhastSystem* Development Technique, PMSF - phenylmethylsulfonyl fluoride, PR-proteins - pathogenesis related proteins, PVX - potato virus X, PVY - potato virus Y, Rf - relative distance, SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TMV - tobacco mosaic virus, TSWV - tomato spotted wild virus,

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pathogen-specific but is determined by the type of reaction displayed by the host plant, indicating that they are of host origin (Van Loon 1987).

Several PR-proteins have recently been characterized as chitinases (Garcia Breijo *et al.* 1990) and  $\beta$ -1,3-glucanases (Kombrink *et al.* 1988). Others have an unknown function but include possible non-specific defense activities against microbial pathogens and insect pests (Fritig *et al.* 1987) or they might be involved in acquired resistance to virus infection (Bol *et al.* 1990). In addition to their pathogen-related induction, some of them are present in healthy tobacco flowers, irrespective of microbial attack or other stress (Lotan *et al.* 1989).

In this paper we investigate the production of PR proteins in *Capsicum* leaves that have been systematically infected with cucumber mosaic virus (CMV), which produces mosaic symptoms but no necrosis.

### Material and methods

Pepper plants (*Capsicum annuum* L. cv. Smith-5) were grown in 20 cm pots containing a standard sterilized potting compost under greenhouse conditions. Prior to sowing, the seeds were disinfected with  $100 \text{ g l}^{-1} \text{ Na}_3\text{PO}_4$  for 15 min.

At 2 months, when they had 10 - 12 leaves (counting only leaves that were at least 5 cm long) the plants were mechanically inoculated with CMV suspension, using carborundum as abrasive. The inoculum was originally obtained from leaves of pepper plants from Region of Murcia, with a light-dark green mosaic. Those leaves were analyzed to determine the kind of virus by ELISA test and shown to be cucumber mosaic virus (CMV) (positive) and tobacco mosaic virus (TMV), potato virus Y (PVY), potato virus X (PVX) and tomato spotted wild virus (TSWV) (negative). From this analysis and from the symptoms on the host plant, the virus isolate was characterized as CMV type I. The virus was maintained in *Nicotiana tabacum* cv. Xanthi-nc and was inoculated as crude sap extracts in 0.01 M phosphate buffer pH 7.1,  $5 \text{ g l}^{-1}$  sodium bisulphite and  $5 \text{ g l}^{-1}$  sodium diethyldithiocarbamate in the proportion of 0.5 g fresh tissue per  $4 \text{ cm}^3$  extraction buffer. The healthy plants, sham-inoculated with the phosphate buffer and carborundum only, were used as control. All the plants were kept in growth chambers with a photoperiod of 14 h ( $175 \mu\text{mol}(\text{photons})\text{m}^{-2} \text{ s}^{-1}$ ) at  $25 \text{ }^\circ\text{C}$  and 65 % relative humidity for the incubation period.

Sham-inoculated and CMV-infected leaves harvested 10 d after inoculation were homogenized in a pH 2.8 buffer containing 84 mM citric acid, 32 mM  $\text{Na}_2\text{HPO}_4$ , 32 mM 2-mercapto ethanol, 6 mM L-ascorbic acid and 0.5 mM PMSF using  $2 \text{ cm}^3$  buffer per 1 g fresh tissue. Tissues were homogenized at  $4 \text{ }^\circ\text{C}$  for 2 min at maximum speed in a *Sorvall Omnimixer*, filtered through muslin and centrifuged at  $30\,000 \text{ g}$  for 30 min at  $4 \text{ }^\circ\text{C}$ . The supernatants were dialyzed overnight (cut off: 12 000 D) against 8.4 mM citric acid, 0.6 mM L-ascorbic acid and 0.5 mM PMSF solution at  $4 \text{ }^\circ\text{C}$ .

The protein concentrations of the extracts were determined by Lowry *et al.* (1951) method and then stored at  $-20 \text{ }^\circ\text{C}$ . Frozen samples were thawed in a thermostatic bath at  $35 \text{ }^\circ\text{C}$  and centrifuged at  $6\,000 \text{ g}$  for 20 min at  $4 \text{ }^\circ\text{C}$ . The supernatants were concentrated on *Centriprep 10 concentrators* (Amicon, USA) to  $30 \text{ mg cm}^{-3}$  and

analyzed by electrophoresis. The proteins were extracted 10 d after inoculation and analyzed by one and two dimensional PAGE, using the *PhastSystem*.

The *PhastSystem* separation and development unit, *PhastGel Gradient 8-25*, buffer strips for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and for native-PAGE of acidic proteins were from *Pharmacia* (Uppsala, Sweden). For PAGE of basic proteins under native conditions, buffer strips were made according to *PhastSystem* Application File No. 300 (*Pharmacia*). The low  $M_r$  calibration kit (*Pharmacia*) was used to estimate  $M_r$ . The other analytical grade reagents were from *Serva* (Heidelberg, Germany), *Sigma* (St Louis, USA) and *Fluka* (Buchs, Switzerland). Water was of *Milli-Q* quality.

Samples for native-PAGE were applied to gels using a 8/1 sample applicator after dipping the pins in 2 mm<sup>3</sup> (= V) sample drops on *Parafilm*. The first-dimensional native PAGE of acidic proteins was run using the programmed conditions of 400 V, 2.5 W and 15 °C of temperature. Step 1 (prerun) was run at 10.0 mA for 10 Vh. Step 2 (sample application) was run at 1 mA, the sample applicator falling 2 after 0 Vh (accumulated 10 Vh) and rising after 2 Vh (accumulated 12 Vh). Step 3 (separation) was run at 10 mA for 268 Vh. For native-PAGE of basic proteins, the programmed conditions were; Step 1 (separation) 200 V, 10.0 mA, 2.5 W, 15 °C and 280 Vh. The sample applicator fell in step 1 after 1 Vh and rose after 10 Vh. For 2D-PAGE, strips about 3 mm wide were cut from native gels and incubated for equilibration for 2.5 min at room temperature in 10 cm<sup>3</sup> of the following solution: 0.112 M Tris, 0.112 M sodium acetate, 10 g l<sup>-1</sup> dithiothreitol and 25 g l<sup>-1</sup> SDS. They were then incubated for another 2.5 min. in 10 cm<sup>3</sup> of the same solution containing 0.260 M iodo-acetamide and adjusted to pH 6.4. Strips were applied directly onto the stacking zone of *PhastGel 8-25 %* gradient and the  $M_r$  markers applied using a *Gilson* micropipette after the strip had been placed in position. The second-dimensional electrophoresis was run with a setting of 250 V, 3.0 W and 15 °C. Step 1 (prerun) was run at 2.5 mA during 5 Vh. Step 2 (sample application) run at 5 mA during 5 Vh. The first direction gel strips were applied to the stacking zone at 0 Vh and removed after 10 Vh. The relative molecular mass markers were applied to the stacking zone with a *Gilson* micropipette at the beginning of step 2. Step 3 (separation) was run at 10 mA during 80 Vh.

Gels were stained according to *PhastSystem* Development Technique (PDT), *Pharmacia*, Uppsala, Sweden. Native gels were stained with Coomassie Blue (*PhastGel Blue R*, *Pharmacia*) according to PDT File No 200 and SDS gels were silver stained according to PDT File No 210.

## Results

Native-PAGE of acidic proteins revealed one principal spot (Fig. 1) clearly absent in the sham-inoculated control, with  $R_f$  0.83. Submission to 2D-PAGE under SDS-denaturing conditions revealed two principal spots (Fig. 2A) suggesting that the band which separated in the first direction contained two proteins, PR1 ( $M_r$  15.3 kD) and PR2 ( $M_r$  29 kD). These may be charge isomers or oligomers but a more extensive

comparison of their electrophoretic mobilities is required to test this.

Native-PAGE of basic proteins revealed at least four bands absent in the sham-inoculated controls. These correspond to Rf 0.48, 0.65, 0.79 and 0.80. Another two bands, named x and y with Rf of 0.32 and 0.68, respectively, can be detected in the sham-inoculated leaves although in very weak concentrations (Fig. 1.). They may not therefore be strictly "novel" proteins in infected plants, but they are to be mentioned for their spectacular increase after CMV infection.

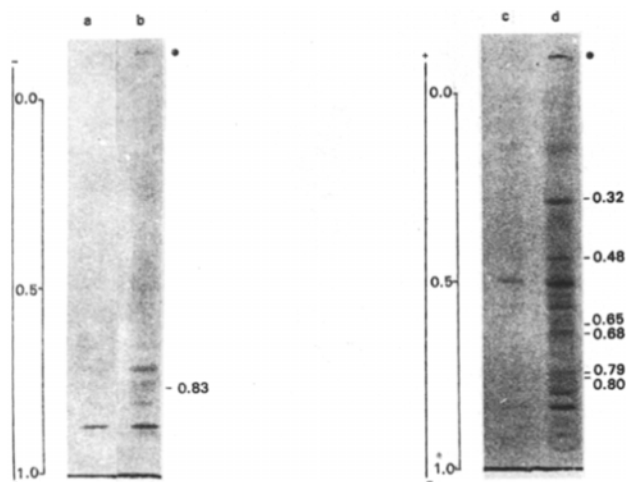


Fig. 1. Coomassie staining of *PhastGel Gradient 8-25* native PAGE of soluble sham-inoculated leaf extracts (lanes *a* and *c*) or CMV-infected leaves (lanes *b* and *d*) 10 days after inoculation. Lanes *a* and *b* correspond to native-PAGE of acidic proteins and lanes *c* and *d*, to native-PAGE of basic proteins. The PR-proteins are labelled as described in the text.

Table 1. Basic PR-proteins in leaves of *Capsicum annuum* after infection with CMV.

Protein	2D-PAGE $M_r$ [kD]	Native-PAGE [Rf]
PR1a	15.9	0.65
PR1b	15.0	0.79
PR1c	15.9	0.80
X	28.7	0.32
Y	20.2	0.65
PR2	27.0	0.68
PR3	36.3	0.48
PR4	48.8	0.48

The proteins were detected by 2D-PAGE using *PhastSystem*

With two dimensional electrophoresis, the Rf 0.48 and 0.65 bands separated into two new spots (Fig. 2B). As with the acid proteins, it was impossible to identify them

as oligomers or charge isomers. The  $M_r$  of these proteins is given in Table 1.

### Discussion

At least eight major PR-proteins (two acidic and six basic) were identified by 2D-PAGE in the soluble extracts from *Capsicum annuum* leaves infected with CMV. We have included only those PR-proteins which are clearly present 10 days after inoculation and which cannot be detected in sham-inoculated leaves, used here as the control. This excluded proteins which could be induced by wounding. Thus, the PR-proteins identified must be due to viral infection.

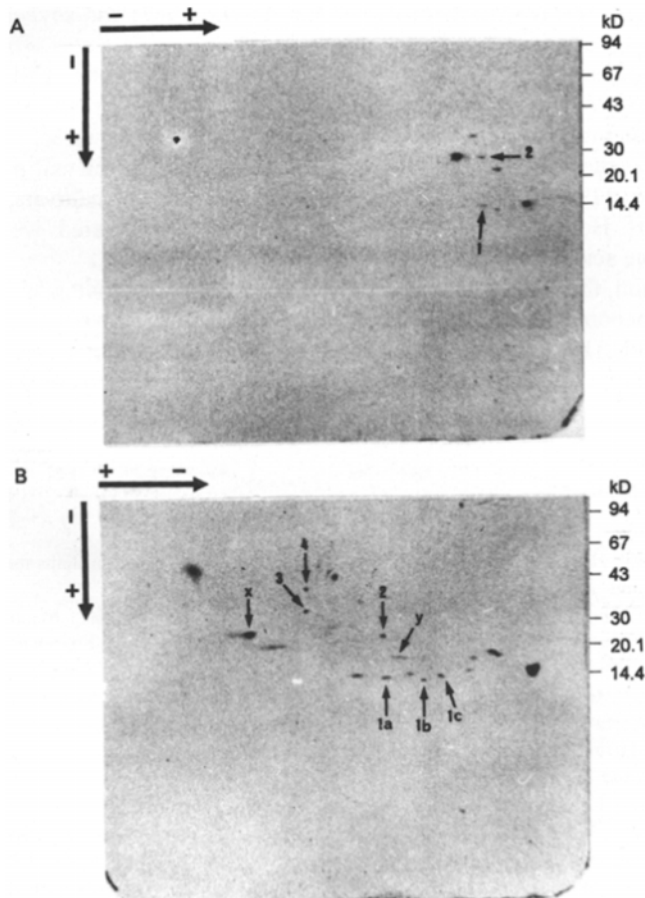


Fig. 2. Silver staining of *PhastGel Gradient 8-25* 2D-PAGE under SDS-denaturing conditions of acidic (A) and basic (B) proteins. First dimension: *PhastGel Gradient 8-25*, native conditions of CMV-infected leaves (see Fig. 1 lane *b* and *d*). The  $M_r$  markers are indicated. The PR-proteins are labelled as described in text.

Two of the eight PR-proteins are acidic. There are great differences in the number of acidic PR-proteins found in the several plants reported in the bibliography. A high number of acidic-PR-proteins have been found as a response to necrotic viral infections in tobacco and *Chenopodium quinoa* (Parent and Asselin 1984), potato (Parent and Asselin 1987) and soybean (Roggero and Pennazio 1989). Four acidic PR-proteins have been identified in petunia cultivars (Nicoud *et al.* 1987) and only one acidic PR-protein was found in alfalfa (Hooft Van Huijsduijnen *et al.* 1986), in *Nicotiana glutinosa* (Parent and Asselin 1984) and in cucumber (Metraux *et al.* 1988).

Six of the eight PR-proteins were basic. To the authors' knowledge, basic PR-proteins have only been described for tomato (Granell *et al.* 1987), tobacco (Van Loon and Gerritsen 1989), potato (Parent and Asselin 1987) and soybean (Roggero and Pennazio 1990).

In pepper plants we know of no reference to PR-proteins after CMV infection. Tobias *et al.* (1989) have reported the interaction between *Capsicum annuum* and tobacco mosaic virus and they found four major PR-proteins with  $M_r$  of 11, 18, 21 and 28 kD, together with several other minor bands. The experimental procedures of Tobias *et al.* (1989) and ours differ as regards pepper cultivars, virus and electrophoresis. However, if the PR-proteins obtained are compared, we find two of similar  $M_r$ , one acidic of 29 kD and the other basic of 27 kD.

In conclusion, the synthesis of PR-proteins as a response to pathogenic infection is a common phenomenon, but the number of proteins obtained vary substantially in range and kind. This is due not only to the different experimental procedures used but also to the type and sensitivity of the pathogens and their interaction with the host plant.

## References

- Bol, J.F., Linthorst, H.J.M., Cornelissen, B.J.C.: Plant pathogenesis-related proteins induced by virus infection. - *Annu. Rev. Phytopathol.* **28**: 113-138, 1990.
- Fritig, B., Kauffmann, S., Dumas, B., Geoffroy, P., Kopp, M., Legrand, M.: Mechanism of the hypersensitive reaction of plants. - In: *Plant Resistance to Viruses*. (Ciba Foundation Symposium 133.) Pp. 92-108. John Wiley & Sons, Chichester 1987.
- Garcia Breijo, F.J., Garro, R., Conejero, V.: C<sub>7</sub>(P32) and C<sub>6</sub>(P34) PR proteins induced in tomato leaves by exocortis viroid infection are chitinases. - *Physiol. mol. Plant Pathol.* **36**: 249-260, 1990.
- Gianinazzi, S., Martin, C., Vallee, J.C.: Hypersensibilité aux virus, température et protéines solubles chez le *Nicotiana xanthi* nc. Apparition de nouvelles macromolécules lors de la repression de la synthèse virale. - *Compt. rend. Acad. Sci. Paris* **270**: 2383-2386, 1970.
- Granell, A., Belles, J.M., Conejero, V.: Induction of pathogenesis-related proteins in tomato by citrus exocortis viroid, silver ion and ethephon. - *Physiol. mol. Plant Pathol.* **31**: 83-90, 1987.
- Hooft van Huijsduijnen, R.A.M., Ablas, S.W., De Rijk, R.H., Bol, J.F.: Induction by salicylic acid of pathogenesis-related proteins and resistance to alfalfa mosaic virus infection in plant species. - *J. gen. Virol.* **67**: 2135-2143, 1986.
- Kombrink, E., Schroeder, M., Hahlbrock, K.: Several "pathogenesis-related" proteins in potato are 1,3- $\beta$ -glucanases and chitinases. - *Proc. nat. Acad. Sci. USA* **85**: 782-786, 1988.
- Lotan, T., Ori, N., Fluhr, R.: Pathogenesis-related proteins are developmentally regulated in tobacco flowers. - *Plant Cell* **1**: 881-887, 1989.

- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. - *J. biol. Chem.* **193**: 265-275, 1951.
- Metraux, J.P., Streit, L., Staub, T.: A pathogenesis-related protein in cucumber is a chitinase. - *Physiol. mol. Plant Pathol.* **33**: 1-9, 1988.
- Nicoud, S., Dumas, E., Gianinazzi, S.R.F. White, R.F.: Hypersensitive reaction in *Petunia*. I. Protein changes in the intercellular fluid from *P. axillaris* cv. "M" leaves inoculated with TMV. - *J. Phytopathol.* **119**: 122-130, 1987.
- Parent, J.G., Asselin, A.: Detection of pathogenesis-related proteins (PR or b) and other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. - *Can. J. Bot.* **62**: 564-569, 1984.
- Parent, J.G., Asselin, A.: Acidic and basic extracellular pathogenesis-related leaf proteins from fifteen potato cultivars. - *Phytopathology* **77**: 122-130, 1987.
- Roggero, P., Pennazio, S.: The extracellular acidic and basic pathogenesis-related proteins of soybean induced by viral infection. - *J. Phytopathol.* **127**: 274-280, 1989.
- Roggero, P., Pennazio, S.: Two-dimensional polyacrylamide gel electrophoresis of extracellular soybean pathogenesis-related proteins using *PhastSystem*. - *Electrophoresis* **11**: 86-90, 1990.
- Tobias, I., Fraser, R.S.S., Gerwitz, A.: The gene- for -gene relationship between *Capsicum annuum* L. and tobacco mosaic virus: effects on virus multiplication, ethylene synthesis and accumulation of pathogenesis-related proteins. - *Physiol. mol. Plant Pathol.* **35**: 271-276, 1989.
- Van Loon, L.C.: Disease induction by plant viruses. - *Adv. Virus Res.* **33**: 205-255, 1987.
- Van Loon, L.C., Gerritsen, Y.A.M.: Localization of pathogenesis-related proteins in infected and non-infected leaves of Samsun NN tobacco during the hypersensitive reaction to tobacco mosaic virus. - *Plant Sci.* **88**: 131-140, 1989.
- Van Loon, L.C., Van Kammen, A.: Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN". - *Virology* **40**: 199-211, 1970.

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