BIOLOGIA PLANTARUM 35 (1): 87-93, 1993

The extracellular acidic and basic virus-elicited proteins of cucumber cotyledons

V. REPKA, E. KOLLEROVÁ and M. SLOVÁK

Institute of Experimental Phytopathology and Entomology, Slovak Academy of Sciences, 900 28 Ivanka pri Dunaji, Slovakia

Abstract

Ten major host-encoded pathogenesis-associated proteins have been found in extracts of intercellular sap of cucumber cotyledons infected with tobacco necrosis virus. By native and two-dimensional SDS-polyacrylamide gel electrophoresis, five major acidic virus-elicited proteins have been identified and classified on the basis of their molecular mass in three groups: group 1 included one protein of 14 kD; group 2, one protein of 28 kD and group 3, three proteins of 37-40 kD. Another five virus-elicited proteins were basic, and were again classified in three groups: group 1, included two proteins of 15-16 kD; group 2, two proteins of 22-23 kD; group 3, one protein of 40 kD.

Introduction

Many plants show a hypersensitive response to virus infection, which often results in necrotic local lesions at the primary infection site (Ponz and Bruening 1986). In a number of plant species, virus infection is accompained by the synthesis of a set of acid-soluble proteins called pathogenesis-related proteins or PRs (Van Loon 1990). These products, however, are coded by the host and not by the virus.

For the first time, these PRs belonging to the group of defense-related proteins, have been found in tobacco plants reacting hypersensitively to TMV infection (Van Loon and Van Kammen 1970). To date, PRs have been observed in plants belonging to different families, *e.g. Solanaceae, Fabaceae, Graminae.*

Besides pathogenic micro-organisms, numerous non-infectious treatments have also been shown to induce PRs (Asselin *et al.* 1985), but differences between both types of stress stimuli exist with respect to the rates of accumulation and amounts of PRs induced.

Received 16 September 1991, *accepted* 6 January 1992.

Abbreviations used: M_r - relative molecular mass, PAGE - polyacrylamide gel electrophoresis, PRs - pathogenesis-related proteins, TMV - tobacco mosaic virus, TNV - tobacco necrosis virus

Acknowledgements: The authors thank Mrs. Judita Blahutiaková for her skilful photographical assistance.

Pathogenesis-related proteins were characterized by their acidic nature (Van Loon 1976), their resistance to proteases (Van Loon 1982), and their extracellular location (Parent and Asselin 1984). More recently, however, basic homologues to some of acidic PRs have been investigated. In tobacco, these proteins are not secreted into the intercellular space of the leaf but accumulate in the vacuoles (Bol *et al.* 1990)

As far as we know, to date only one acidic pathogenesis-associated protein have been found in infected cucumbers (Andebrhan *et al.* 1980, Tas and Peters 1977, Wagih *et al.* 1983, Metraux *et al.* 1988). However, there is no report on the occurence of another acidic and basic virus-elicited proteins in cucumber.

In the present work we report the occurence of a new set of extracellularly localized acidic and basic proteins in cucumber plants locally infected with virus giving necrosis.

Material and methods

Cucumber plants (*Cucumis sativus* L. cv. Laura) were grown from seed in a temperature-controlled greenhouse (20 - 32 °C) under natural light. At an age of *ca.* 7 d, the two primary leaves were dusted using carborundum as an abrasive and then rubbed with a foam pad containing a leaf homogenate from cucumber plants infected with TNV. Plants treated similarly with a homogenate of uninfected leaves were used as controls.

The intercellular fluid (IF) from virus-infected and control leaves was isolated by vacuum infiltration and subsequent extraction. Freshly harvested leaf material (10-50 g batches) was washed and infiltrated with glass distiled water *in vacuo* (10 min at *ca.* 2 kPa). After allowing excess water to drip off, the infiltrated leaves were blotted dry with filter paper, then carefully rolled into 50 ml centrifuge tubes. The tubes contained plastic marbles at the bottom to provide space for the extract. The tubes were centrifuged at 1000 *g* for 20 min. The IF thus obtained was used immediately or freeze-dried and stored at -20 °C. Protein concentration in the extracts was determined colorimetrically according to Bradford (1976) with bovine serum albumin as the standard.

Discontinuous polyacrylamide gel electrophoresis (PAGE) of acidic proteins was performed at 4 °C under native conditions according to Laemmli (1970), with the exception that SDS was omitted in all buffers. The sample buffer did not contain 2-mercaptoethanol. PAGE was performed using 4 % stacking gel and 10 % separation gel in tubes (0.5 × 12 cm). Each lane was loaded with equal amounts of protein (50 or 100 µg) and bromphenol blue as marker. PAGE of basic proteins was performed under native conditions according to Reisfeld *et al.* (1962) but omitting the stacking gel and using a 15 % separating gel in tubes. Each tube was loaded with the same amount of protein as mentioned above and methyl green as marker. PAGE was run at 4 °C for 8 - 10 h under constant current (4 mA per tube). Tube gels were stained with Coomassie Brilliant Blue R-250 and destained with a mixture of water: acetic acid: methanol (6:1:3, v/v/v) and stored in 7 % acetic acid.

Two-dimensional (2D) PAGE was carried out on cylindrical gels after native PAGE in the first dimension. The cylindrical gels were equilibrated with SDS

sample buffer (63 mM Tris-HCl pH 6.8, containing 2 % SDS, 5 % 2-mercaptoethanol and 10 % glycerol) in a test tube at room temperature for 45 min., followed by immersion in boiling water for 5 min. The gel pieces were mounted on top of a second-dimension 10-15 % (for basic gels) or 12-15 % (for acidic gels) gradient gel slab containing 0.1 % SDS, followed by electrophoresis according to Laemmli (1970). A protein standard kit (*BIO-RAD*, SDS-PAGE Standards - *LMW*) was used as MM marker. Staining and destaining of gel slabs were carried out as mentioned for native gels.

Results

The acidic protein patterns of IF extracts from healthy or 7-d TNV-inoculated leaves showed both qualitative and quantitative changes. As compared to extracts from healthy leaves, 4 additional protein bands appeared in IF from virus-infected leaves (Fig. 1 A) then electrophoresed in the presence of SDS (second dimension) revealed five major protein spots (Fig. 2 A), absent in the healthy controls (Fig. 2 B). Some minor spots in both infected and control extracts were detectable only by visual inspection of the gels.

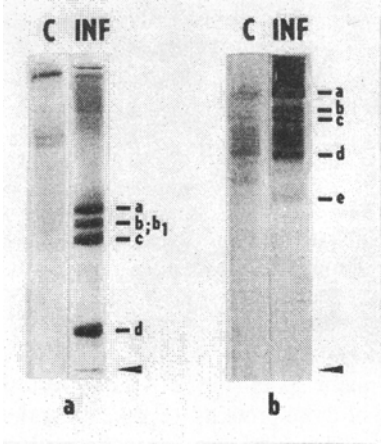

Fig. 1. Electrophoretic patterns of acidic (a) and basic (b) IF proteins of cucumber from non-infected control leaves (lanes C) and from TNV-infected leaves (lanes INF) after separation by native PAGE. Acidic and basic proteins from intercellular fluids were run on 10 % and 15 % polyacrylamide gels, respectively. Gels were loaded with 50 µg total proteins. The virus-elicited proteins are labelled in order of increasing mobility. The position of dye marker is indicated by arrowheads.

The acidic virus-elicited proteins were consecutively named in order of increasing electrophoretic mobility under native conditions. According of increasing molecular mass, the acidic proteins were grouped into three groups: group 1, b_1 (14.7 kD); group 2, d (28.1 kD); group 3, c (37.1 kD), b (38 kD) and a (40.7 kD). Among quantitative changes, a spot X was visible with difficulty in gel from healthy control

whereas it was visible as a prominent spot in gel from virus-infected leaves (Figs. 2 A and B).

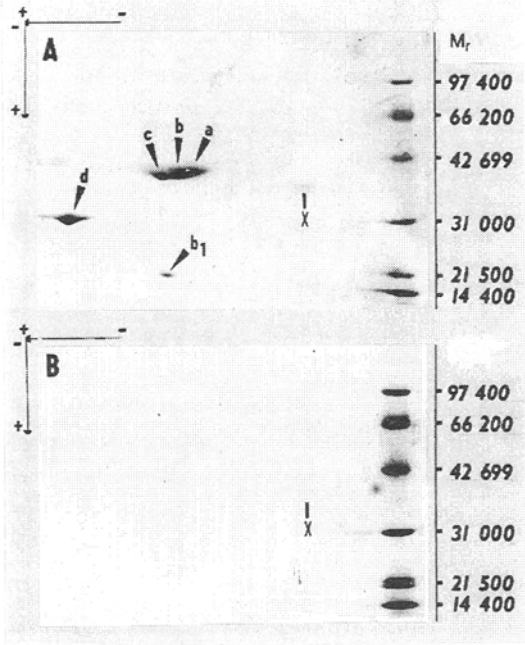

Fig. 2. Two-dimensional electrophoretic patterns of (A) acidic virus-elicited proteins from TNV-infected cucumber leaves, and (B) a comparable fraction from non-infected control leaves. First dimension (horizontal arrow), electrophoresis in 10 % native gels (see. Fig. 1 a); second dimension (vertical arrow), 12-15 % gradient SDS-containing gel. The position of the M_r markers are indicated. The acidic virus-elicited proteins are labelled as described on the text.

The pattern of basic proteins revealed five additional spots in the IF of TNV inoculated leaves as compared to healthy controls (Fig. 1 b). Two-dimensional PAGE in the presence of SDS gave also five major spots (Fig. 3 A), absent in the healthy controls (Fig. 3 B) and indicated the M_r of these proteins, relative to markers. The basic virus-elicited proteins were grouped as for acidic proteins in three groups: group 1, b (15.5 kD) and e (16.5 kD); group 2, d (22.9 kD) and c (23.4 kD); group 3, a (40.7 kD). Surprisingly, two additional bands, named Y and Z (estimated M_r *ca.* 22-24 kD) were visible in healthy control (Fig. 3 B), but fully absent in the IF of virus-inoculated leaves (Fig. 3 A).

Discussion

By two-dimensional PAGE at least ten major virus-inducible proteins have been detected in the intercellular fluids from cucumber leaves reacting hypersensitively to TNV.

Five out of the ten virus-elicited proteins are acidic. A number of studies have shown that specific host-encoded proteins (probably PRs) accumulated in both infected and osmotically stressed cucumber cotyledons (Andebrhan *et al.* 12980, Tas and Peters 1977, Gessler and Kuc' 1982) and in the true leaves of cucumber infected with phytopathogens (Gessler and Kuc' 1982). More recently Metraux *et al.* (1988) have identified a 28 000 PR-protein in cucumber leaves, but their PR-protein was somewhat larger than those previously reported in cotyledons (22 kD, Gessler and Kuc' 1982) and in leaves (16 kD, Wagih *et al.* 1983) of cucumber. It is difficult to say whether the protein identified by different authors is the same protein or not. We surmise an existence of a set of proteins what was confirmed by the two findings. On the one hand, whereas 22 000 and 16 000 proteins were only detected locally, a 28 000 protein was detected as far as five leaves away from the site of infection. On the other hand, none of previously identified acidic cucumber pathogenesis-associated proteins had a counterpart in a set of proteins detected in our work. There is only one exception. Metraux *et al.* (1988) also found that their PR-protein (28 kD) is a chitinase. In preliminary study on function of purified acidic virus-elicited proteins of cucumber we also confirmed that the protein designated as d (Fig. 2 A) had chitinolytic activity. Therefore, we suggest that it is the same or closely related (chitinase isozyme) protein.

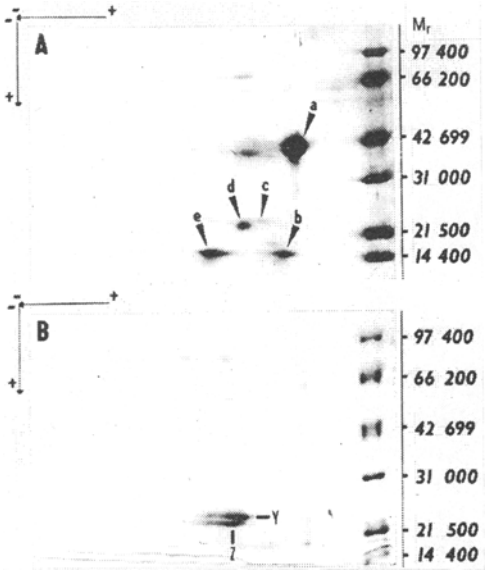

Fig. 3. Two-dimensional electrophoretic patterns of (A) basic virus-elicited proteins from TNV-infected cucumber leaves, and (B) a comparable fraction from non-infected control leaves. First dimension (horizontal arrow), electrophoresis in 15 % native gels (see Fig. 1 b); second dimension (vertical arrow), 10-15 % gradient SDS-containing gel. The position of the Mr markers are indicated. The basic virus-elicited proteins are labelled as described on the text.

The converse also could be true. A great number of acidic PR-proteins as a response to nectrotic viral infections have been found in different plants. This may be due to difference either in experimental procedure employed or plant cultivar used. As already reported for tobacco (Parent and Asselin 1984) the extraction by IF method or purification allowed to obtain a greater number of PR-proteins than conventional extraction, *e.g.* by acidic McIlvaine buffer (pH 2.8).

Five major virus-elicited proteins of cucumber were basic. As far as we know there is no literature report about identification of a basic set of proteins elicited by pathogenesis or other forms of stress in cucumber. To date, basic PR-proteins have been described only for a few plant species (*e.g.* tobacco, tomato, potato and soybean).

At the present we do not know whether new acidic and basic virus-elicited proteins just identified in our work belong to the group of PR-proteins. Antoniw *et al.* (1980) proposed to call PR-protein those host-encoded proteins induced only in pathological or related situation (for the nomenclature see Van Loon 1990). Recently we have found that at least some of our proteins share a common properties with PR-proteins (*e.g.* range of MM, chitinase and β-1,3-glucanase activity *etc.*). The need of further studies of biochemical, serological and functional properties new identified virus-elicited proteins with the well characterized PR-proteins could be very useful. These works are now in progress.

References

Andebrhan, T., Coutts, R.H.A., Wagih, E.E., Wood, R.K.S.: Induced resistance and changes in the soluble protein fraction of cucumber leaves locally infected with *Colletotrichum lagenarium* or tobacco necrosis virus. - Phytopathol. Z. **98**: 47-52, 1980.

Antoniw, J.F., Ritter, C.E., Point, W.S., Van Loon, L.C.: Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. - J. gen. Virol. **47**: 79-87, 1980.

Asselin, A., Grenier, J., Cote, F.: Light-influenced extracellular accumulation of b proteins in *Nicotiana green* tisue induced by various chemicals or prolonged floating on water. - Can. J. Bot. **63**: 1276-1283, 1985.

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.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Gessler, C., Kuc', J.: Appearance of a host protein in cucumber plants infected with viruses, bacteria and fungi. - J. exp. Bot. **33**: 58-66, 1982.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Metraux, J.P., Streit, L., Staub, T.: A pathogenesis-related protein in cucumber is a chitinase. - Physiol. mol. Plant Pathol. **33**: 1-9, 1988.

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 citrus. - Can. J. Bot. **62**: 564-569, 1984.

Ponz, F., Bruening, G.: Mechanism of resistance to plant viruses. - Annu. Rev. Phytopathol. **24**: 355-383, 1986.

Reisfeld, R.A., Lewis, J., Williams, D.E.: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. - Nature **195**: 281-283, 1962.

Tas, P.L., Peters, D.: The occurrence of a soluble protein (E_1) in cucumber cotyledons infected with plant viruses. - Neth. J. Plant Pathol. **83**: 5-12, 1977.

Van Loon, L.C.: Specific soluble leaf proteins in virus-infected tobacco plants are not normal constituents. - J. gen. Virol. **309**: 375-379, 1976.

Van Loon, L.C.: Regulation of changes in proteins and enzymes associated with active defence against virus infection. - In: Wood, R. (ed.): Active Defense Mechanisms in Plants. Pp. 247-273. Plenum Press, New York 1982.

Van Loon, L.C.: The nomenclature of pathogenesis-related proteins. - Physiol. mol. Pathol. **37**: 229-230, 1990.

Van Loon, L.C., Van Kammen, A.: Polyacrylamide disc electrophoresis of the soluble leaf protein from *Nicotiana tabacum* var. Samsun and Samsun NN. II. Changes in protein constitution after infection with tobacco mosaic virus. - Virology **40**: 199-211, 1970.

Wagih, E.E., Raftopoulos, A.E., Archer, S.A., Coutts, R.H.A.: Characterisation of an apparently novel soluble fraction found in pathogen and osmotically-stressed cucumber cotyledons. - Phytopathol. Z. **107**: 233-243, 1983.