INTRA- AND EXTRACELLULAR ISOFORMS OF PR-3 CLASS CHITINASE IN VIRUS-INFECTED CUCUMBER PLANTS

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Summary. – Cucumber (*Cucumis sativus* L. *cv.* Laura) contains three different isoforms of chitinase (EC 3.2.1.14), thought to be involved in the defense against a pathogen. Using a highly specific rabbit antiserum raised against a predominant, extracellularly localized, virus-inducible acidic chitinase (p28), two additional enzyme isoforms with differential mode of compartmentalization were identified. Immunoblot analysis of the fractionated plant extracts separated by denaturing and native (anodic and cathodic) polyacrylamide gel electrophoresis (PAGE) revealed that the chitinase p25 (M_r 25.5 K), is a basic, etxracellular isoform and the chitinase p24 (M_r 24.6 K) is an acidic, probably intracellular isoform of the enzyme.

Key words: cucumber; chitinase isoforms; immunoblot analysis; tobacco necrosis virus

Introduction

Plants reacting hypersensitively to infection with viruses, bacteria, or fungi start to accumulate a set of host-encoded proteins that exert their function either in the cytoplasm, the cell wall, or the intercellular space of the leaf. As a group, these proteins are believed to be involved in a plethora of defense mechanisms resulting in a systematically acquired resistance of the plant (Collinge and Slusarenko, 1987; Van Loon 1988; Ward *et al.*, 1991). Those of these induced proteins that are excreted into the extracellular space of the leaf are commonly known as "pathogenesis-related" (PR) proteins. Several classes of PR-proteins have been characterized in more detail and some of them well shown to have a chitinase activity.

A circumstantial evidence suggests that chitinases represent structurally diverse groups of enzymes, which differ in their physical properties, activities and location in plant compartments (Boller, 1989; Shinshi *et al.*, 1990). Besides defense response, chitinases also exhibit a complex developmental and hormonal regulation (Memelink *et al.*, 1987; Chrispeels 1991). In the case of cucumber, inoculation of leaves with tobacco necrosis virus (TNV) induces one major, extracellularly localized, acidic chitinase p28 (M_r 28 K; Repka and Slováková, 1992). Based on a structural gene analysis as well as on comparison of partial amino acid sequences, this cucumber enzyme belongs to the third class chitinases including the lysozyme/chitinases from papaya, *Hevea*, *Parthenocissus*, and *Rubus* (Métraux *et al.*, 1989).

Because PR-proteins are commonly analysed in alkaline gel systems, the basic chitinases have probably been overlooked so far. With the exception of the basic chitinase isoforms occuring in tobacco, there is a little information available regarding the basic chitinase isoenzymes in other plant species.

To address the question of the occurence of various chitinase isoforms accumulated in virus-infected cucumber, we have used the antiserum raised against the prominent virus-inducible extracellular chitinase (p28).

Abbreviations: EDTA = ethylenediamine tetraacetic acid; ICF = intercellular fluid; PAGE = polyacrylamide gel electrophoresis; PR = pathogenesis related; PVP = polyvinylpyrrolidone; SDS = sodium dodecyl sulphate; TNV = tobacco necrosis virus

Materials and Methods

Plant material. Cucumis sativus L. cv. Laura plants were grown from seed in soil in a greenhouse at 20 – 30°C. Plants were watered to saturation daily.

Inoculation of plants. Fully expanded 7-day-old cucumber cotyledons were dusted using carborundum and infected with a partially purified suspension of TNV as described elsewhere (Repka and Slováková, 1994). Leaves of control plants were inoculated similarly except that the virus isolation buffer or distilled water were used instead of the virus. Plant samples were harvested daily during the seven consecutive days and frozen immediately.

Protein extraction. To obtain intracellular fluid (ICF) extracts, cotyledons were processed by the vacuum infiltration procedure as described in detail previously (Repka *et al.*, 1993). The ICF-extracts were centrifugally concentrated using a Centriprep-3 concentrator (Amicon) according to the manufacturer's protocol. The leaves from which the ICF was eluted were used to obtain extracts depleted of ICF. The leaves were ground in a mortar with pestle with about 1 g of quartz sand and an appropriate amount of TRISEPAC buffer (50 mmol/l Tris HCl pH 8.0, 500 mmol/l sucrose, 1 mmol/l ethylenediamine tetraacetate (EDTA), 0.2% polyvinylpyrrolidone (PVP), 6 mmol/l ascorbic acid and 0.1% cysteine) and centrifuged at 15,000 x g for 20 mins at 4°C. The same procedure was used for freshly harvested cotyledons to obtain total extracts. Supernatant fractions were kept frozen at -20°C.

Protein concentration was measured according to Bradford (1976) using bovine serum albumin as the standard.

PAGE. Discontinuous PAGE (10%) of acidic proteins was performed at 4°C under native conditions according to Leammli (1970) with the exception that sodium dodecyl sulphate (SDS) was omitted from all buffers and the sample did not contain β-mercaptoethanol. The basic proteins were separated under native conditions on 10% gels following the original protocol of Reisfeld *et al.* (1962). A small drop (5 µl) of 5% solution of basic fuchsin was used as the tracking dye. PAGE under denaturing conditions (SDS-PAGE) was performed on 1.5 mm slab gels using a 4% stacking gel and a 12.5% separating gel according to Laemmli (1970). Low-molecular mass standards (Bio-Rad) were co-electrophoresed on the same gel.

Detection of chitinase activity after SDS-PAGE. SDS-PAGE was performed with 12.5% polyacrylamide gels containing 0.1% SDS and 0.01% glycol chitin as substrate. The substrate was synthesized from glycol chitosan (Sigma) following the modified protocol of Trudel and Asselin (1989). Samples from healthy and TNV-infected cotyledons were boiled for 3 mins with 15% sucrose and 2.5% SDS in 125 mmol/l Tris.HCl pH 6.8) without β-mercaptoethanol. Bromphenol Blue (0.01%) was used as the tracking dye and gels were run in the standard way (Laemmli, 1970). After electrophoresis, proteins were renatured by incubation of the gel for 17 hrs at 37°C with reciprocal shaking in 100 mmol/l sodium acetate buffer pH 5.2 containing 1% Triton X-100 (Aldrich Chemicals) purified through a mixed-bed resin deionizing column (AG 501-X8, Bio-Rad). Then the chitinase activities were detected by 0.01% Calcofluor White M2R (Sigma) dissolved in 500 mmol/l Tris.HCl pH 8.9. Lytic zones were visualized by placing the gels on a Chromato-Vue C-62 transilluminator (UVP Products, Cambridge, UK) and photographed using GDS 5000 gel documentation system (UVP Products, Cambridge, UK) equipped with UV-haze and yellow filter. The chitinase activities were seen as dark bands against the fluorescent background. Molecular mass markers (Bio-Rad) were co-electrophoresed in the same gel and silver-stained according to Blum *et al.* (1987).

Immunoblot analysis. Immediately after PAGE was completed, the proteins from gels were electroblotted to a nitrocellulose membrane (TM-NC4, 0.45 µm, Hoefer Scientific Instruments, Inc., San Francisco, USA) at 4°C for 24 hrs at 50 mA using 0.7% acetic acid or 40 mmol/l Na-phosphate buffer pH 6.5 as a transfer medium. Following the transfer, membranes with proteins separated under native conditions were heated at 80°C for 8 hrs to inactivate endogenous peroxidases. The membranes were blocked in Blotto solution containing 5% non-fat dry milk (Repka and Slováková, 1994) and incubated with rabbit anti-p28-chitinase antibody diluted 1:200. The blots were further processed according to the basic procedure (Repka and Jung, 1995). The immunopositive signals were detected using 0.03% 3,3-diaminobenzidine (Amresco, Inc., Solon, OH, USA) and 0.03% $NiCl_2/CoCl_2$ as an intensifier. Molecular mass standards (Bio-Rad) on blots were stained with 0.1% Amido Black.

Results

Identification of chitinase isoforms in virus-infected leaves

ICF-extracts from healthy and TNV-infected cucumber cotyledons were first analysed for the presence of the chitinase activities in SDS-PAGE gels containing glycol chitin as substrate. In ICF-extracts from healthy leaves we did not find any chitinase activity, while in those prepared from cucumber leaves 7 days after infection with TNV at least 7 chitinase isoforms were observed (Fig. 1). Besides three prominent chitinase isoforms with an apparent M_r of 28 K (p28), 25 K (p25) and 24 K (p24), the ICF-extracts from TNV-infected leaves showed four bands with faint chitinase activity. Apparent M_r of these activities ranged from 38 K to 50 K.

The expression and identification of the different virus-inducible cucumber chitinases was also investigated by comparing specific immunoreactivities between healthy and virus-inoculated cotyledons. The immunoblots from leaf homogenates showed strongly positive signals only in the total or ICF-extracts prepared from TNV-inoculated cotyledons (Fig. 2, lanes A, B, C and D).

Altogether three immunoreactive bands were identified in leaf extracts prepared from TNV-infected plants after SDS-PAGE (Fig. 2). The antiserum specifically cross-reacted besides with the former acidic chitinase p28 also with other two proteins designated as p25 and p24 with apparent M_r of 25.5 K and 24.6 K, respectively. Moreover, no positive signals were obtained with other chitinase isoforms revealed in the extract using the activity staining of the gel.

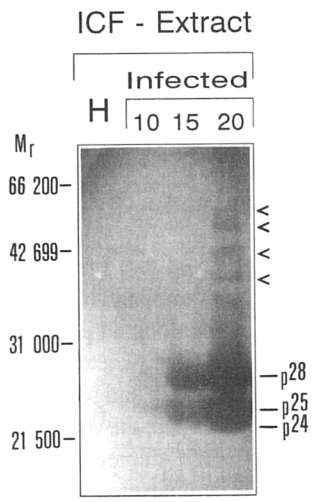

Fig. 1
Chitinase activity in healthy and TNV-infected cucumber cotyledons
ICF-extracts from healthy (H, 20 μg of total proteins) and TNV-infected cucumber cotyledons (10, 15 and 20 μg of total proteins) were subjected to SDS-PAGE. Four minor chitinase isoforms are marked by arrowheads. Numbers on the left refer to M_r of standards.

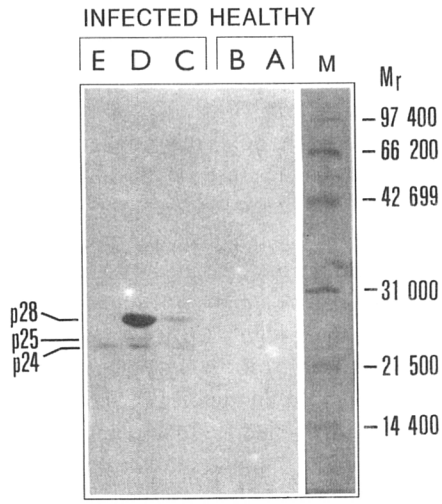

Fig. 2
Immunoblot analysis of chitinase isoforms in healthy (A, B) and TNV-infected (C, D, E) cucumber cotyledons
Samples corresponding to total plant extract (A, C), ICF-extract (B, D) and ICF-depleted extract (E) containing equal amounts of protein (10 μg/lane) were loaded on SDS-PAGE gel. As a probe, the antiserum raised against the predominant chitinase isoform p28 was used. Lane M denotes M_r of standard.

Localization of chitinase isoforms

The localization of the different chitinase isoforms was further studied by comparing the patterns of distribution of individual chitinases present in fractionated plant extracts 7 days after infection (Fig. 2, lanes C, D, E). The most intensive immunoreactive band of former chitinase p28 was present in the ICF-fraction, confirming its extracellular localization. The corresponding immunopositive band, albeit of about ten-fold lower intensity of the signal was observed in the total plant extract, while no signal was detected in the ICF-depleted extract.

The isoform designated as p25 was immunodetected strictly in the ICF-fraction indicating its extracellular compartmentalization. In contrast, the chitinase isoform p24 was localized mainly in the ICF-depleted extract indicating an intracellular localization, although the same amount of the enzyme was detected in the ICF-fraction and its traces in the total plant extract.

Time-course of accumulation of different chitinase isoforms

To confirm a final destination of different chitinase isoforms, the products accumulated in fractionated plant extraxt at 7 consecutive days were analyzed using various PAGE procedures. The immunoblot analysis of ICF proteins separated under denaturing conditions revealed that all three chitinase isoforms were accumulated in the ICF-fraction (Fig. 3A). Starting from day 3, p28 was the most abundant protein present in the extracellular space of the cotyledon. The isoforms p25 and p24 started to accumulate from day 4 and 5, respectively.

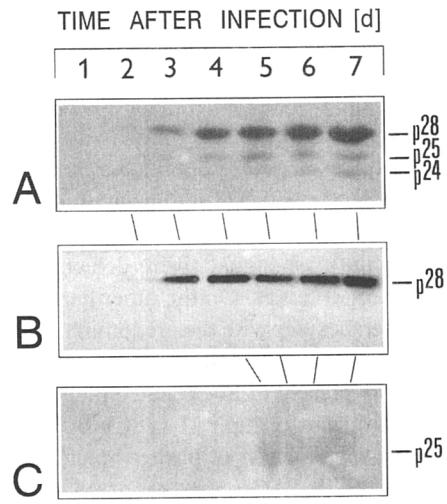

Fig. 3
Immunoblot analysis of the time course of accumulation of chitinase isoforms in TNV-infected cucumber cotyledons
ICF-extracts (A, B) and total plant extract (C) from TNV-infected cucumber cotyledons were analysed. Equal amounts of protein (10 μg) were loaded per lane of the gel and separated on 12.5% SDS gel (A), 10% native anodic gel (B) and 10% native cathodic gel (C). As a probe, the rabbit anti-p28 serum was employed.

The immunoblot analysis of the anodically separated ICF-fraction under native conditions revealed the presence of a single immunoreactive band, corresponding to the major extracellular chitinase p28 (Fig. 3B). In contrast, the cathodical separation of the total plant extract under native conditions also showed the presence of a single immunoreactive band (Fig. 3C). Since the time-course pattern obtained under the latter conditions was the same as that of chitinase isoform p25 separated under denaturing conditions, the observed immunopositive signal corresponded to this chitinase. The third chitinase identified, p24, must be an acidic, intracellularly localized isoform since no immunoreactive signal was present either in anodically separated ICF-extract or in cathodically separated total plant extract.

Discussion

Three prominent chitinase isoforms with M_r ranging from 24.6 K to 28 K and four faint chitinase isoforms (M_r 38 K – 50 K) were identified in hypersensitively reacting cotyledons of TNV-infected cucumber using the activity staining of the SDS-PAGE gel. As far as we know, this is the first report on a diversity of chitinase forms in cucumber although a predominant, extracellularly localized isoform (p28) has independently been described (Boller and Métraux, 1988; Repka and Slováková, 1992). We have used the highly specific antiserum raised against this isoform as a tool for the study of the isoenzyme diversity and compartmentalization of this class of plant hydrolases.

Two kinds of results were obtained from immunoblott experiments using the anti-p28 serum and fractionated plant extracts prepared from healthy and virus-infected plants. First, positive chitinase signals were detected only in extracts from infected leaves. On the other hand, the antiserum cross-reacted besides with the predominant isoform p28 also with other two proteins but not with four chitinase isoforms of high M_r present in fractionated plant extracts. The presence of chitinases of high M_r in ICF of TNV-infected leaves is surprising since M_r of purified plant chitinases as determined by SDS-PAGE was reported to be around 30 K (Boller, 1985). These types of chitinases have been reported often in microorganisms (bacteria and fungi) but rarely in higher plants (Boller, 1989). The strawberry chitinases estimated at 42 K, 47 K and 80 K are likely to be of plant origin since similar bands were found also in healthy strawberry leaf extracts El Ghaouth *et al.*, 1991).

Although not identified so far, the existence of various isoforms of PR-3 class chitinase in cucumber was suggested by Métraux *et al.* (1989). They have reported an isolation of a complementary DNA encoding the cucumber chitinase (M_r 28 K) which is analogical to one of ours. Eight positive clones were isolated from a cDNA library using the synthetic probes. Since all 8 clones had the same nucleotide sequence, it very probably reflects the high degree of serological relatedness of chitinase isoforms identified by us. However, a critical discrepancy is the number of clones and isoforms identified. This could be partially explained by a genomic Southern blot analysis, suggesting that the cucumber genome may contain other genes highly-related to the chitinase gene.

In this study, we subjected to a preliminary analysis the localization of chitinase isoforms in TNV-infected leaves. The immunoblot analysis of fractionated plant extracts provided an evidence that these isoforms were differentially compartmentalized. While the acidic p28 and basic p25 isoforms were strictly extracellularly localized, the third isoform (p24) was highly accumulated in the ICF-depleted extract and thus must have been intracellularly compartmentalized. The results with the total leaf homogenate as well as with the ICF-fraction showed a weak band corresponding to p24 isoform. The appearance of p24 isoform in these extracts was probably due to a contamination, i.e. it could have been released from the dead cells in the hypersensitively responding areas of the leaf. This statement is also in accord with the results of the time course study, confirming that p24 isoform was first detected in ICF-fraction 5 days after infection, i.e. at the time when the tissue necrosis associated with hypersensitive response was very well developed.

Although there is no direct evidence for p24 isoform, studies on isolated protoplasts as well as a vacuoles showed vacuolar localization of the intracellular chitinases (Boller and Vogeli, 1984; Boller and Métraux, 1988; Neuhaus *et al.*, 1991). The vacuolar and/or extracellular compartmentalization of plant chitinases were confirmed by the findings of Métraux *et al.* (1989) who claimed that all eight clones had signal sequence involved in an effectively operating sorting system. According to Boller (1985) most plant chitinases including those localized vacuolarly are basic proteins. In contrast to this, we showed in this study that intracellular chitinase p24 is an acidic protein since no corresponding band was observed either in ICF-extract or total plant extract separated by anodic or basic PAGE gel system, respectively. Obviously, further experimental work will be necessary to determine the actual localization of the identified chitinase isoforms in TNV-infected plant tissues.

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