IDENTIFICATION AND PARTIAL CHARACTERIZATION OF β-1,3-GLUCANASE FROM VIRUS-INFECTED CUCUMBER COTYLEDONS

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Summary. – One of the five "pathogenesis-related" (PR) proteins known to accumulate in *Cucumis sativus* L. *cv.* Laura and to react hypersensitively to tobacco necrosis virus (TNV) was shown to to have β-1,3-glucanase activity. The TNV-induced acidic β-1,3-glucanase activity increased 6-fold after infection and had extracellular localization and estimated M_r of 25,700. The β-1,3-glucanase activity was investigated with a new method of activity staining using a conjugated substrate in overlay gels. By using the antiserum against tobacco β-1,3-glucanase purified to homogeneity, a close serological relationship was demonstrated between cucumber and tobacco β-1,3-glucanases on immunoblots.

Key words: pathogenesis-related proteins; β-1,3-glucanase; *Cucumis sativus* L.; tobacco necrosis virus; polyacrylamide gel electrophoresis; immunoblot analysis

Introduction

Plants, unlike animals, have no immune system, and yet they succesfully fight an infection by use of both constitutive and induced defense mechanisms. Upon infection of plants with different kinds of pathogens (*e.g.* viroids, viruses, bacteria or fungi), the development of symptoms is frequently accompanied by the accumulation of soluble host-encoded proteins (van Loon and van Kammen, 1970; Gianinazzi *et al.*, 1980, Ahl *et al.*, 1981, Camacho-Henriquez and Sänger, 1982, Legrand *et al.*, 1987). Since their appearance at first could only be related to pathological situation they were named "*pathogenesis-related*" proteins (PR proteins, Antoniw *et al.*, 1980). The induction of PR proteins upon pathogen attack has been demonstrated in more than 20 different species, suggesting that this represents a general feature of the active defense response of plants (for a review, see Carr and Klessig, 1990, Bowles, 1990). Although the functions of many PR proteins are still unknown, recently it has been demonstrated that some of them are β-1,3-glucanases (Kauffmann *et al.*, 1987).

Plant β-1,3-glucanases or laminarinases (EC 3.2.1.39) hydrolyze various β-1,3-glucans that could act as elicitors of host defense responses (Keen and Yoshikawa, 1983; Hahn *et al.*, 1989). There are several suggestions that plant β-1,3-glucanases may be components of a general defense strategy against pathogen invasion (Vögelli *et al.*, 1988; Benhamou *et al.*, 1989; Meins and Ahl, 1989).

Besides pathogen attack, the expression of these enzymes is regulated, at least in part, at the mRNA level by the plant hormones auxin, cytokinin, and ethylene (Boller, 1985; Cabello *et al.*, 1994). At the protein level, they are classified in two main groups, acidic and basic enzymes, although three distinct classes have been established based on the structural analysis of their genes (Linthorst, 1991). The basic isoforms are described as vacuolar, while the acidic group includes extracellular enzymes (van den Bulcke *et al.*, 1989). Specific roles in the plant defense response have been pro-

Abbreviations: HR = hypersensitive response; ICF = intercellular fluid; PAGE = polyacrylamide gel electrophoresis; PR = pathogenesis-related; SDS = sodium dodecyl sulphate; TNV = tobacco necrosis virus

posed for these two groups of β-1,3-glucanases (Mauch and Stahelin, 1989).

We have reported previously that the expression of a specific set of acidic soluble proteins is highly induced in response to virus infection resulting in a hypersensitive reaction (HR) (Repka *et al.*, 1993). In spite of the great wealth of data available on PR proteins, thus far no biological function has been demonstrated for all these proteins. Therefore, in order to obtain a better insight into the possible role of the PR proteins expressed in our model system, we have studied and partially characterized a protein with proposed β-1,3-glucanase activity potentially involved in the defense response of hypersensitively reacting cucumber plants.

Materials and Methods

Plant material. Cucumber plants (*Cucumis sativus* L. *cv.* Laura) were grown in organic pots (diameter 10 cm, Jiffy Products, Oslo, Norway) in a greenhouse at 25°C and relative humidity of 60%. All plants were watered to saturation daily.

Inoculation of plants. At an age of approximatelly 7 to 10 days, plants were transferred to a growth chamber (22°C) and the cotyledons were dusted with carborundum and inoculated with a suspension of TNV as described previously (Repka and Slováková, 1994). Control plants were treated either with virus isolation buffer or were untreated.

Protein extraction. Virus-inoculated leaves were harvested on days 1-7 after inoculation and soluble extracellular proteins were extracted by the vacuum infiltration procedure (Repka *et al.*, 1993). Intercellular fluids (ICF) were collected by centrifugation at 10,000 x g for 10 mins and concentrated using a microconcentrator (Microcon-3, Amicon, Witten, FRG). The samples were freeze-dried and stored at -20°C until use.

Protein determination was done according to Bradford (1976) using bovine serum albumine (fraction V, Calbiochem) as standard.

β-1,3-glucanase activity was assayed in concentrated ICF extracts colorimetrically according to the original protocol of Frič and Wolf (1994), using carboxymethyl-pachyman (β-1,3-glucan) conjugated with Remazol Brilliant Blue (Sigma) as substrate. All enzyme activities were measured in two independent assays using Beckman DU-8 spectrophotometer. The enzyme activity was expressed as the change in the absorbance at 620 nm.

Polyacrylamide gel electrophoresis 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). The same system was used for native PAGE, except that SDS was omitted. Proteins in gels were stained either with Coomassie Blue R-250, Amido Black or silver (Blum *et al.*, 1987).

Activity staining of endo-β-1,3-glucanase in gel after PAGE was done using laminarin-Remazol Brilliant Blue (RBB) as substrate in agarose overlay gel (Sock *et al.*, 1990). After electrophoresis, the separating gel buffered with 1.5 mol/l Tris-HCl pH 8.8 was first incubated 2 x 10 mins in 150 ml of 1 mol/l Na-acetate buffer pH 5.2 at 2°C on an orbital shaker. For detection of β-1,3-glucanase, the separating gel was then immediately laid on a laminarin-RBB agarose gel and incubated in a thermostat at 37°C until the laminarin-RBB degradation zones indicating enzyme activity developed optimally. After incubation, the agarose gel was destained overnight in 95% ethanol. Gel areas with β-1,3-glucanase activity appeared as colorless spots on a transparent, dark blue background. The gel was photographed on a light box using an orange filter to obtain the highest contrast.

Immunoblot analysis. Proteins separated on SDS-PAGE gels were electroblotted at 4°C for 20 hrs at 50 mA to a nitrocellulose membrane (TM-NC4, 0.45 μm, Hoefer Scientific Instruments, San Francisco, CA, USA) in 40 mmol/l Na-phosphate buffer pH 6.5. β-1,3-glucanases were detected immunologically on the blots using a rabbit antiserum against tobacco β-1,3-glucanase diluted 1:500. The blots were blocked with 5% non-fat dry milk (Blotto) and processed according to the standard procedure (Repka and Slováková, 1994). Serological reactions were detected by a colour method using 0.03% 3,3-diaminobenzidine (Amresco, Solon, OH, USA) and 0.03% $NiCl_2/CoCl_2$ as an intensifier. Low-molecular mass protein standards (BIO-RAD) were stained with 0.1% solution of Amido Black and destained with the mixture of 45% methanol and 7% acetic acid.

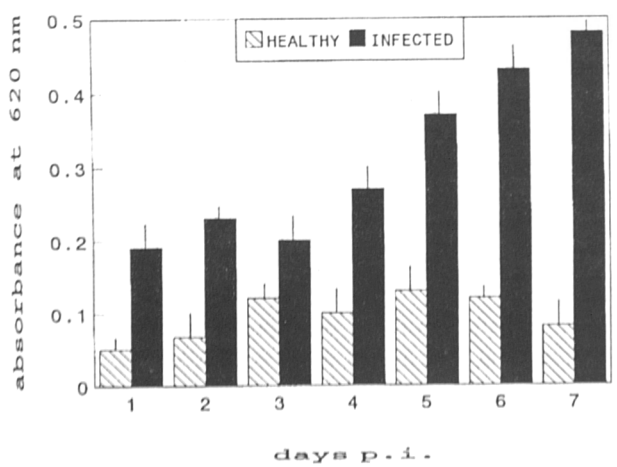

Fig. 1
β-1,3-glucanase activity in TNV-infected and healthy cucumber leaves
The enzyme activity is expressed in A_{620} units. The data are mean values from 2 separate measurements. Vertical bars represent maximum S.E. values.

Results

Induction of β-1,3-glucanase activity during HR of cucumber plants

The activity of β-1,3-glucanase was low in untreated plants but was stimulated strongly after TNV infection. The activity showed approximately 6-fold increase by the 7th day of infection (Fig. 1).

Identification of β-1,3-glucanase in cucumber

In a search for proteins responsible for β-1,3-glucanase activity in TNV-infected cucumber leaves, we subjected protein extracts to PAGE. The *in gel* activity staining of cucumber anionic β-1,3-glucanase using an overlay gel containing dye-labelled laminarin revealed the presence of only one band with glucanase activity (Fig. 2). The corresponding protein present in ICF extracts of virus-infected cucumber plants was clearly visible as one band in silver stained gel. The unique band was lightly visible also in Coomassie Blue stained gels but only by visual inspection of the gel.

Serological relationships between cucumber and tobacco β-1,3-glucanases

An antiserum prepared against the purified tobacco acidic β-1,3-glucanase was used in an immunoblot experiment presented in Fig. 3. The estimated M_r of β-1,3-glucanase polypeptide in ICF from virus-infected cucumber leaves was 25,700. There was no visible serological cross-reaction of the antiserum with other proteins present in ICF extracts prepared from TNV-infected, as well as from the control cucumber leaves.

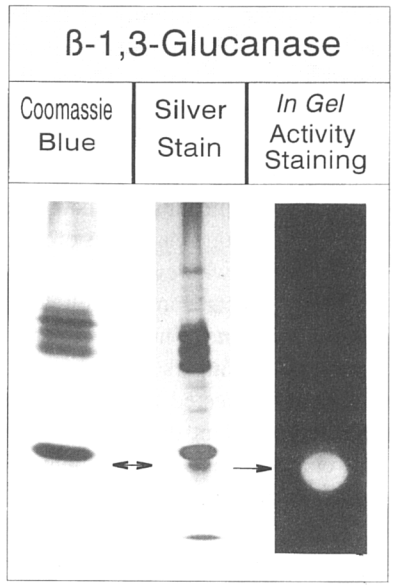

Fig. 2
Native PAGE of proteins extracted from TNV-infected cucumber cotyledons
The lanes were loaded with 50 μg, 3 μg and 50 μg of total ICF proteins and stained with Coomassie Blue, silver stain and *in gel* activity stain, respectively. Horizontal arrows refer to the corresponding protein with β-1,3-glucanase activity.

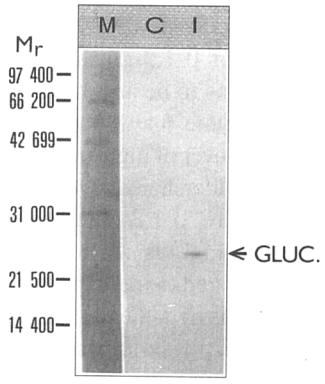

Fig. 3
Immunoblot analysis of ICF extracts from TNV-infected and healthy cucumber leaves with antiserum against tobacco β-1,3-glucanase
ICF extracts (50 μg of proteins per lane) from control (C) and TNV-infected (I) leaves were subjected to SDS-PAGE and immunoblot analysis. Lane M contained M_r standards stained with Amido Black.

Discussion

This study was undertaken to give additional information to our previous report on cucumber PR proteins (Repka *et al.*, 1993). Among the five acidic PR proteins described, several are PRXs and chitinases (Repka and Slováková, 1994; Métraux *et al.*, 1988; Repka and Slováková, 1992). While the hydrolase, chitinase, is relatively well characterized to date, there is no substantial information available regarding the β-1,3-glucanase. To our knowledge, this is the first report on the identification and partial characterization of cucumber β-1,3-glucanase.

Our results indicate that an appreciable amount of β-1,3-glucanase activity is present in the extracts from hypersensitively reacting cucumber cotyledons. Based on the time course experiments, it is evident that the kinetics of induction of this enzyme is very similar to that described for β-1,3-glucanase activity in *Nicotiana glutinosa* (Moore and Stone, 1972) and *N. tabacum cv.* Samsun NN (Kauffmann *et al.*, 1987) infected with tobacco mosaic virus.

In this study, we used an *in gel* activity staining technique to identify and visualize the position of polypeptides with β-1,3-glucanase activity on polyacrylamide gels. Using this method, we showed that a cucumber protein with M_r of 25,700 displays high β-1,3-glucanase activity. In this respect, the existence of only one polypeptide with glucanase activity in cucumber is surprising, since Coté *et al.* (1991) detected multiple bands on genomic immunoblots probed with λFJ1 insert corresponding to the amino-terminal domain of the tobacco PR-2c (glucanase) protein. These results suggest the presence of a small family of β-1,3-glucanase (PR-2) genes in cucumber. The discrepance between our results and those described by Coté *et al.* (1991) could

be explained by a higher sensitivity of the Southern blot analysis, used by the latter. Alternatively, it is possible that in TNV-infected or control plants some specific β-1,3-glucanase isozymes are expressed at very low level just at the limit of the *in gel* activity staining method using the dye-labelled substrate. An existence of a proposed multiple isozyme forms of β-1,3-glucanase in cucumber is indirectly supported by the time course analysis of glucanase accumulation observed by us, where an activity, albeit low, was documented also in extracts from visually healthy plants.

Since β-1,3-glucan is one of major cell wall components in infected or stressed plants, some specific β-1,3-glucanase activity may be connected with the deposition of this enzyme during normal plant development. Fraser (1981) provided strong evidence for a developmental regulation of PR proteins in leaves of healthy tobacco plants during flowering. Fraser's results together with those of Lotan *et al.* (1989) and Memelink *et al.* (1990) on the floralspecific expression of PR genes suggest that PR-2 class proteins (β-1,3-glucanases) play a role in normal plant development.

We showed that antiserum raised against tobacco PR-2 (glucanase) protein cross-reacted with β-1,3-glucanase but not with any other PR protein of cucumber. These data confirm the serological relationship at least between these two proteins (tobacco and cucumber glucanases).

A deposition of callose (β-1,3-glucan) has been previously reported in many host-virus combinations (Shimomura and Dijkstra, 1975), however, the functional significance of high levels of β-1,3-glucanase activity in virus-infected tissues remains to be answered. One of the potential roles of this enzyme may be its involvement in the processing and/or turnover of high-molecular β-glucans and elicitors (Cline and Albersheim, 1981; Darvill and Albersheim, 1984). Therefore, β-1,3-glucanases are likely to be a part of the fundamental resistance to pathogen invasion and not necessarily related to incompatible host-virus interactions. To examine this hypothesis, additional immunological, biochemical and physiological studies are urgently needed. They are just in progress.

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