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Short note

New acidic chitinase isoforms induced in tobacco roots by vesicular-arbuscular mycorrhizal fungi

Eliane Dumas-Gaudot[1], Valentin Furlan[2], Jean Grenier[3], and Alain Asselin[3]

[1] Laboratoire de Phytoparasitologie, INRA-CNRS, Station de Génétique et d'Amélioration des Plantes, INRA, BV 1540, F-21034 Dijon Cedex, France
[2] Station de Recherches, Agriculture Canada, 2560, Boulevard Hochelaga, Sainte-Foy, Quebec G1V 2J3, Canada
[3] Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Quebec G1K 7P4, Canada

Summary. Chitinase activities have been compared in tobacco roots (*Nicotiana tabacum* cv. Xanthi nc) infected by the pathogenic fungus *Chalara elegans* or three species of vesicular arbuscular mycorrhizal (VAM) fungi: *Glomus versiforme*, *G. intraradix* and *G. fasciculatum*, using native polyacrylamide gel electrophoresis (PAGE). All previously known acidic chitinase isoforms were stimulated in roots by the pathogenic fungus and by the VAM fungi, while two new acidic chitinase isoforms were specifically induced in response to the endomycorrhizal association. After separation in sodium dodecyl sulphate polyacrylamide denaturing gels (SDS-PAGE) under non-reducing conditions, the estimated apparent molecular mass for these additional acidic chitinase isoforms from VAM-colonized samples was 33 kDa, compared to 30 kDa for the main activity stimulated in *C. elegans*-infected root extracts.

Key words: Tobacco roots – *Chalara elegans* – *Glomus* spp. – Chitinases – PR proteins

Introduction

Evidence for weak or transient activation of some defence responses during early stages of the vesicular arbuscular mycorrhizal (VAM) symbiosis has been reported (Spanu and Bonfante-Fasolo 1988; Dumas et al. 1989, 1990; Bonfante-Fasolo and Perotto 1990; Gianinazzi 1991), with special emphasis on chitinases (Spanu et al. 1989; Dumas et al., to be published). Plant chitinases (EC 3.2.1.14) are potential antifungal hydrolases (Schlumbaum et al. 1986; Roberts and Selitrennikoff 1988; Mauch et al. 1988a, b) which have also been investigated in various host-pathogen interactions (Pegg and Young 1982; Roby and Esquerré-Tugayé 1987; Mauch et al. 1988a, b). Recently, several chitinases have been identified as pathogenesis-related (PR) proteins (Legrand et al. 1987; Kombrick et al. 1988; Metraux et al. 1988; Nasser et al. 1988; Joosten and De Wit 1989; Trudel et al. 1989).

Using detection techniques for chitinase activity after native polyacrylamide gel electrophoresis (PAGE) or denaturing conditions (SDS-PAGE), the complete set of chitinase isoforms stimulated in virus-infected tobacco leaves or present in some of the healthy tissues has been well established (Trudel et al. 1989). Up to now, we have failed to detect acidic chitinases in endomycorrhizal tobacco roots by Western blots, while their occurrence has been clearly demonstrated using the same analytical approach in tobacco roots infected by the pathogenic fungus *Chalara elegans* (Dumas et al. 1989; Tahiri-Alaoui et al. 1990). The aim of the present work was to re-examine VAM-colonized and *C. elegans*-infected root extracts using new electrophoretic detection techniques for chitinase activity.

Materials and methods

VAM-infected *N. tabacum* cv. Xanthi nc. was obtained by sowing tobacco seeds in trays filled with Turface (calcined montmorillonite clay; Imcore Division, Illinois, USA) and inoculating with leek roots infected by *Glomus versiforme* (Karst.) Berch, *G. intraradix* (Schenck & Smith) and *G. fasciculatum* (Thaxter) (Gerdeman & Trappe emend. Walker & Koske) as previously described (Despatie et al. 1989). Plants were grown in a controlled-environment room (17/23° C ± 1° C, 16-h photoperiod at 300 $\mu mol\, m^{-2}\, s^{-1}$, 60% relative humidity). They were watered weekly with a low phosphorus Long Ashton solution ($HPO_4^{2-} = 44\, mg\, l^{-1}$) at the rate of $4\, l\, m^{-2}$ of Turface and supplemented with distilled water. Control plants were grown in the same way, except that the inoculum consisted of non-mycorrhizal leek roots. For pathogen infection, tobacco plants were individually transplanted into Turface and inoculated 2 weeks later with *C. elegans* (Isolate 84-1 from the Tobacco Institute, Bergerac, France) as previously described (Tahiri et al. 1990). Control plants received only water.

Control and VAM-colonized roots were analysed after 10 weeks [average of 20–30% root colonization determined as described by Kormanick et al. (1980)] and after 14 weeks (average of 50–60% root colonization). Control and *C. elegans*-infected roots were sampled 4, 7 and 11 days after inoculation. Root homogenates were made by grinding tissue in liquid nitrogen and the result-

Offprint requests to: E. Dumas-Gaudot

ing powders were resuspended in 0.1 M sodium phosphate buffer pH 6.5 (1:1 w/v) at 4° C. Homogenates were then centrifuged at 10000*g* for 1 h at 4° C and kept at −20° C. Sample preparation for chitinase activity after PAGE under native conditions for the separation of acidic or neutral proteins (Davis 1964) and under non-reducing denaturing conditions (SDS-PAGE) was performed as previously described (Trudel and Asselin 1989). Renaturation of enzyme activity after SDS-PAGE by incubation in the appropriate buffer for 18 h at 37° C and staining of chitinase activities with Calcofluor White M2R were carried out according to Trudel et al. (1989).

Results

At least six main bands with chitinolytic activity separated in the Davis system at pH 8.9 were observed in the control root extracts (Fig. 1, lanes C1 and C2). These bands had the same electrophoretic mobilities as those of the PR proteins previously identified as chitinases in an intercellular fluid from either virus-infected or chemically treated leaves of tobacco (Trudel et al. 1989) (Fig. 1, bars on the left). Until now, acidic chitinases have not been detected in healthy tobacco roots while two root-specific acidic chitinases were found in cucumber (Trudel et al. 1989; Majeau et al. 1990). In the present case, the occurrence of acidic chitinases in healthy tobacco roots could be related to general stress of the plants (Fig. 1, lane C1). Interestingly, the healthy roots (Fig. 1, lane C2), corresponding to the control of the experiment with *C. elegans*, gave a lower signal for most of the chitinase bands, while a clear stimulation of the chitinase activities was observed after infection with *C. elegans* (Fig. 1, lane C.e). This increase was particularly obvious for the two lower bands (first doublet indicated by arrows in Fig. 1). When tobacco roots were colonized with the three *Glomus* species, an increase in the intensity of most of the chitinase bands already present in non-mycorrhizal roots occurred. Preliminary analysis of endomycorrhizal extracts showed a stronger stimulation of chitinase activities in tobacco, reaching 50–60% root colonization, than in those showing only 20–30% colonization. Electrophoretic patterns of chitinase activities are presented only for extracts corresponding to the maximal level of mycorrhizal infection. The observed increase in chitinase activities from endomycorrhizal roots is particularly obvious with *G. intraradix* and *G. fasciculatum* (Fig. 1, lanes G.i and G.f). Furthermore, two acidic chitinase isoforms were newly detected in extracts from roots colonized by the three *Glomus* species (arrowheads in Fig. 1). The band with the highest electrophoretic mobility seemed to have a stronger chitinase activity.

The same extracts were then analysed for chitinase activities after denaturing SDS-PAGE under non-reducing conditions (Fig. 2). Only one band with an apparent estimated molecular mass of 30 kDa was observed in extracts from healthy roots (Fig. 2, lanes C1 and C2) as well as in those from *C. elegans*-infected roots (lane C.e). This is in agreement with previously reported results obtained with virus-infected tobacco leaves and some healthy tobacco tissues (Trudel et al. 1989). This band appeared as a very faint signal in the healthy roots used as controls for the *C. elegans*-infected plants (Fig. 2, lane C2) compared to healthy roots used in the experiment with the mycorrhizal fungi (Fig. 2, lane C1), confirming the results obtained after PAGE under native conditions. Moreover, a noticeable increase of this 30-kDa migrating band was observed in *C. elegans*-infected roots from 4–11 days of infection. In extracts from roots colonized by the three *Glomus* species, a second band was distinctly detected with an apparent estimated molecular mass of 33 kDa. The induction of this addi-

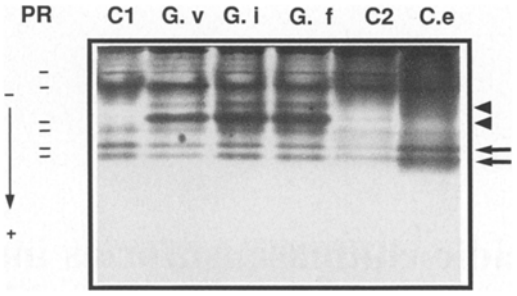

Fig. 1. Chitinase activities after native 15% polyacrylamide gel electrophoresis (PAGE) for acidic proteins (Davis system). Clarified extracts of non-mycorrhizal roots (*C1*) and non-infected (control) plants (*C2*) were compared to *Glomus versiforme* (*G. v*), *G. intraradix* (*G. i*), *G. fasciculatum* (G. f) colonized root and to *C. elegans*-infected root extracts (*C. e*). Samples each contained 30 µg protein. After electrophoresis, the gel was stained with Calcofluor White 2MR and chitinase activities were observed after UV illumination as dark lytic zones against the fluorescent background of glycol chitin (0.01% v/v) embedded in the gel. *Bars* on the left indicate the positions of chitinase activities from an intercellular fluid of chemically induced tobacco leaves (PR). Additional (*arrowheads*) or stimulated (*arrows*) chitinase activities are indicated on the right

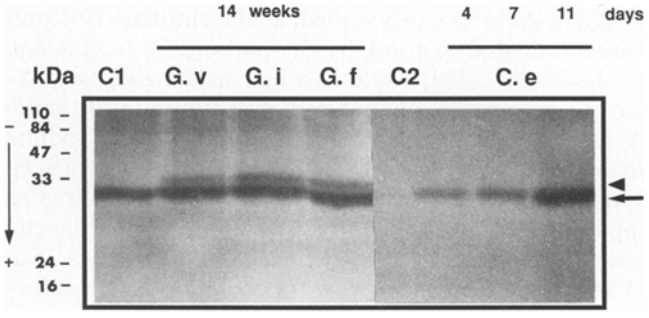

Fig. 2. Chitinase activities after denaturing 15% polyacrylamide gel electrophoresis under non-reducing conditions (SDS-PAGE). Extracts from non-mycorrhizal (*C1*) and non-infected (control) (*C2*) roots were compared to 14-week-colonized roots infected by *G. versiforme* (*G. v*), *G. intraradix* (*G. i*), *G. fasciculatum* (*G. f*) and to *C. elegans*-infected roots (*C. e*) analysed 4, 7 and 11 days after infection. Samples each contained 30 µg protein. Staining was as in Fig. 1. Prestained molecular mass markers (Bio-Rad) are indicated on the left: lysozyme (16 kDa), soybean trypsin inhibitor (24 kDa), carbonic anhydrase (33 kDa), ovalbumin (47 kDa), bovine serum albumin (84 kDa) and phosphorylase (110 kDa). Chitinase activities in mycorrhizal roots are indicated by *arrowheads* (new) or *arrows* (stimulated) on the right

tional chitinase isoform in VAM-colonized roots was confirmed in a two-dimensional gel system (results not shown) as one-dimensional PAGE is not as reliable as two-dimensional gels for distinguishing the various chitinase isoforms (Trudel and Asselin 1989).

Discussion

Several studies have focused on the stimulation of chitinases during plant-microbe interactions (Graham and Graham 1991). However, to our knowledge, only few investigations have been carried out on a single host plant infected by either a pathogenic or a symbiotic fungus (Dumas et al. 1990). Using new analytical PAGE approaches for detection of chitinase activity, we clearly demonstrated for the first time the occurrence of a differential induction of chitinase isoforms according to the infecting fungus. Since both *C. elegans* and the three *Glomus* species contain chitin as a major component of their cell walls (Barnicki-Garcia 1968; Bonfante-Fasolo et al. 1986; Dumas et al., unpublished results), the observed stimulation of chitinase activities is not unexpected. However, we showed that if the activities of the chitinase isoforms previously identified as PR proteins in tobacco (Trudel et al. 1989) are mainly stimulated by the pathogenic fungus *C. elegans*, and to a lesser extent by some stress conditions, at least two additional acidic chitinase isoforms could be related to the mycorrhizal symbiosis. These VAM-related chitinase isoforms could be serologically unrelated to the acidic PR chitinases from tobacco, as no chitinase has been detected in young VAM tobacco root extracts by Western blots when probed with anti-PR chitinases (Dumas et al. 1989). The present results suggest that the diversity of chitinases in healthy versus "biologically stressed" tissue is complex. Selective induction of chitinase isoforms according to the pathovar of *Xanthomonas* has recently been reported in *Brassica* (Dow et al. 1991), but the significance for VAM symbiosis has still to be elucidated.

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