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Chitosanase and chitinase activities in tomato roots during interactions with arbuscular mycorrhizal fungi or *Phytophthora parasitica*

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Abstract

New chitosanase acidic isoforms have been shown in *Glomus mosseae*-colonized tomato roots and their induction, together with the previously described mycorrhiza-related chitinase isoform, has been further corroborated in plants colonized with another *Glomus* species (*G. intraradices*), as well as in tomato roots colonized *in vitro* by *Gigaspora rosea*. The induction of these chitosanase isoforms appears as a specific response to the arbuscular mycorrhizal (AM) symbiosis, and does not correspond to unspecific defence mechanisms, since these isoforms were not induced by the pathogen *Phytophthora parasitica*. Analysis by isoelectrofocusing showed two closely migrating chitinase isoforms, specific to mycorrhizal plants colonized either with *G. mosseae* or *G. intraradices*, and their isoelectric points were estimated to be 4.5 and 4.7. The estimated molecular mass of chitosanases was 20 kDa, and after isoelectrofocusing, the chitosanase activities were detected along the acidic pH range (6.5–3.5). Constitutive and induced isoforms were also investigated during a time-course study. In some experiments, chitin and chitosan were embedded together as substrates in polyacrylamide gels with the aim of studying the capacity of some isoforms to display both chitinase and chitosanase activities. In extracts from plants colonized with either *G. mosseae* or *G. intraradices*, some constitutive chitinases and the previously described mycorrhiza-related chitinase isoform, appeared to display chitosanase activity, while this bifunctional character was not found for the chitinases from non-mycorrhizal tissue, nor in *Phytophthora*-infected plants. These results suggest some diversity in the chitinase activities concerning substrate specificity in mycorrhizal plants. The possible implications of these observations in the functioning of the symbiosis is discussed.

Key words: Arbuscular mycorrhizas, chitinases, chitosanases, *Phytophthora parasitica*, tomato, *Lycopersicon esculentum*.

Introduction

Mycorrhizas are among the most universal, intimate and important symbioses in terrestrial ecosystems. The arbuscular mycorrhizal (AM) type, formed between plants and zygomycetous fungi of the Glomales order, is the most ancient and widespread mycorrhizal symbiosis (Allen, 1996). This symbiosis has been shown to assist the plant to overcome biotic and abiotic stresses (Barea and Jeffries, 1995; Azcón-Aguilar and Barea, 1996). During colonization of the root cortex, co-ordinate cellular development of host cells and the invading fungus achieves a functional mutualistic state. Molecular and biochemical analyses show the elicitation of defence-related molecules during the establishment of the symbiosis, although this elicitation is low and transient in comparison to that of the plant reaction to pathogens (Gianinazzi-Pearson *et al.*, 1996). Increases of certain hydrolase activities, mainly chitinases and β-1,3-glucanases, have been reported for mycorrhizal plants (Spanu *et al.*, 1989; Dumas-Gaudot *et al.*, 1992*b*; Vierheilig *et al.*, 1994; Lambais and Mehdy, 1995; Pozo *et al.*, 1996) and the possible role of these

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enzymes in the regulation of the symbiosis, as well as in plant protection against root pathogens, has been reviewed (Lambais and Mehdy, 1995; Azcón-Aguilar and Barea, 1996; Dumas-Gaudot *et al*., 1996).

Chitinases (EC 3.2.1.14) hydrolyse chitin, a linear homopolymer of β-1,4 linked *N*-acetylglucosamine (GlcNAc), which is one of the major cell wall components of most fungi (Wessels and Sietsma, 1981). They exist as a family of proteins differing in their biochemical characteristics, primary structure and subcellular localization. They can also be differentially regulated, probably playing different roles. Chitinase induction is apparently elicited in higher plants in response to physiological and environmental stress (Collinge *et al*., 1993; Graham and Sticklen, 1994). Although no substrate for this enzyme has been described in plants, it has been suggested that chitinases have a role in plant growth (Patil and Widholm, 1997). A developmental regulation, pointing to a morphogenetic role especially during cell wall disrupting events, such as flowering and reproduction, has also been described (Sahai and Manocha, 1993). However, the induction of chitinases and other hydrolytic enzymes is mainly one of the co-ordinated, often complex and multifaceted, defence mechanisms which are triggered in response to pathogen attack. Disease resistance by hydrolases can occur by hydrolysis of the fungal cell wall or by the liberation of compounds which elicit defence reactions (Sahai and Manocha, 1993).

Less research has been done on chitosanases (EC 3.2.1.99). They are hydrolytic enzymes acting on chitosan, a derivative of chitin partially or fully deacetylated (Monaghan *et al*., 1973). No substrate for chitosanases is known in plants, but chitosan is present in the cell wall of many fungi, including Zygomycetes (Bartinicki-Garcia, 1968; Araki and Ito, 1975). Chitosan and chitosan oligosaccharides can induce defence reactions in plants, including the induction of chitinase, chitosanase and β-1,3-glucanase isoforms (El Ghaouth *et al*., 1994). Several molecular forms of chitosanases have been found in leaf tissues which are stressed chemically or with pathogens (Grenier and Asselin, 1990). Some have been identified as extracellular PR proteins and are present in several acidic and basic forms, with apparent molecular masses ranging from 10 to 23 (El Ouakfaoui and Asselin, 1992*a*). They are believed to have a role in defence against invading pathogens because of their potential to hydrolyse fungal cell wall polysaccharides (Grenier and Asselin, 1990), although they are also regulated according to the organ and/or the developmental stage of the plant (El Ouakfaoui and Asselin, 1992*a*, *b*).

However, distinction between chitinase and chitosanase activity might not be absolute. Some purified plant chitinases can degrade substrates other than chitin, displaying chitosanase (Mayer *et al*., 1996) and/or lysozyme activity (EC 3.2.1.17) (Graham and Sticklen, 1994). Thus, these enzymes can be considered multifunctional.

Differential expression of chitinases in tomato roots in response to AM colonization and *Phytophthora parasitica* infection has been previously reported (Pozo *et al*., 1996). Concerning chitosanases, no activity was detected in seeds, leaves or fruits of non-stressed tomato and only some isoforms were reported in chemically stressed leaves (El Ouakfaoui and Asselin, 1992*a*). Pegg and Young (1982) described in tomato stems a constitutive chitinase with an isoelectric point of 8.5 that was able to cleave internal bonds of chitosan. As far as is known, no study has been carried out on chitosanase activity in tomato roots.

The present research goes deeper in studying the expression of the mycorrhiza-related chitinases by testing different AM fungi, including AM colonization *in vitro*. Chitosanase activities were also investigated in tomato roots during interactions with different AM fungi, both *in vivo* and *in vitro*, and with the fungal pathogen *P. parasitica*. Finally, the possibility that some isoenzymes display both chitinase and chitosanase activities was investigated by means of polyacrylamide gel electrophoresis. The results are discussed in relation to the possible role of these enzymes in the AM symbiosis.

Materials and methods

Plant and fungal material

Experiments were carried out either in containers (*in vivo*) or in Petri dishes (*in vitro*). For the *in vivo* experiments, tomato seeds (*Lycopersicon esculentum* cv. Earleymech) were surface-sterilized with a solution of commercial bleach (50%, v/v) and germinated under sterile conditions on wet filter paper at 28 °C. Seedlings were grown in 750 ml containers containing a sterile mixture of quartz sand and soil (9:1, v/v). The experimental design consisted of four different treatments: control plants (C) and plants inoculated with *Glomus mosseae* (Gm), *Glomus intraradices* (Gi) or *Phytophthora parasitica* (Pp). Each treatment was replicated four times, and three independent inoculation experiments were carried out. Mycorrhizal inoculation was performed by using a soil-sand based inoculum containing fungal propagules and chopped mycorrhizal roots of *Glomus mosseae* Nicol and Gerd (BEG 12) or *Glomus intraradices* Smith and Schenck (BEG 72). All plants received a filtrate ($<20\ \mu$m) of the AM inocula to provide the microbial populations accompanying the mycorrhizal fungi but free from AM propagules.

Inoculation with the pathogenic fungus *Phytophthora parasitica* [synonymous with *Phytophthora nicotianae* van Breda de Haan var. *parasitica* (Dastur) Waterhouse] isolate 201 (kindly provided by P Bonnet, INRA, Antibes, France) was carried out 4 weeks after plants were potted as described above. The pathogen was grown on a malt-agar medium at 25 °C in darkness for 3 weeks, and the inoculum was prepared by washing the growing mycelia with sterile water. The suspension obtained was used to inoculate the corresponding tomato plants, as described by Pozo *et al*. (1996).

For the *in vitro* experiments, tomato seeds were surface-

sterilized for 5 min with a 3.5% calcium hypochlorite solution, rinsed with deionized sterile water and let to dry on sterile paper for 18 h. They were again surface-sterilized for 5 min, rinsed and germinated in Petri dishes. After 1 week, seedlings were placed in large Petri dishes (14 cm diameter, 4 seedlings per dish) on 50 ml of the culture medium described by Crush and Hay (1981), pH 6.8, but containing 0.5% (w/v) activated charcoal. Spores of the AM fungus *Gigaspora rosea* Nicolson and Schenck (BEG 9) were separated from a soil inoculum by wet sieving. They were surface-sterilized for 10 min with a solution containing streptomycin (0.2 mg ml^{-1}), gentamycin (0.1 mg ml^{-1}), chloramine T 5%, and two drops of Tween 20. After that, they were rinsed with deionized sterile water and carefully placed aseptically close to the roots of the tomato seedlings (25 spores per seedling).

Growth conditions and plant harvest

Tomato plants for the *in vivo* experiments were grown in a controlled environment room (25/18 °C day/night temperature, 60% relative humidity, 16 h photoperiod, with a photosynthetic photon flux of 400 μmol photons m^{-2} s^{-1}). They were watered three times per week with Long Ashton nutrient solution (Hewitt, 1966) at one-quarter phosphorus strength. Plants were harvested 4, 6, 8, 10, and 12 weeks after potting, carefully washed in running tap water, rinsed in deionized water and weighed. An aliquot of the root system from each plant was kept for determination of mycorrhizal colonization, and the rest was immediately frozen in liquid nitrogen and stored at −80 °C until protein extraction. For estimation of mycorrhizal establishment, samples from each root system were cleared and stained (Phillips and Hayman, 1970) and the percentage of total root length which was colonized by the AM fungus was measured by the gridline intersect method (Giovannetti and Mosse, 1980). *P. parasitica* infection of tomato roots was evaluated by ELISA using the *Phytophthora* pathogen detection kit Agriscreen (Adgen Diagnostic Systems, Auchincruive, Ayr, Scotland, UK).

For the *in vitro* experiments, cultures were placed up in a growing cabinet (photoperiod 16 h, 17–23 °C) with the lower part of the Petri dish covered with aluminium foil. Plants were kept growing for 8 weeks. After that, selected parts of the root system on which hyphae of the mycorrhizal fungus were well developed were carefully harvested under a dissecting microscope, frozen in liquid nitrogen and stored at −80 °C until used.

Protein extraction and quantification

Frozen roots from plants grown in containers were ground at 4 °C in an ice-chilled mortar with liquid nitrogen and the resulting powder was suspended in 100 mM MacIlvaine (citric acid/Na_2HPO_4) extracting buffer, pH 6.8 (1:1, w/v). Crude homogenates were centrifuged at 15 000 *g* for 30 min at 4 °C and the supernatant fractions were kept frozen at −20 °C. For the *in vitro* experiments, protein extraction was carried out following the same protocol except that roots were directly frozen and ground in Eppendorf tubes with small pestles, due to the small amount of available material. Protein contents were determined by the method of Bradford (1976) using BSA as standard.

Electrophoresis and enzymatic assays

All extracts (10 μg of proteins per sample) were analysed by 15% (w/v) polyacrylamide gel electrophoresis (PAGE) under native conditions according to Davis (1964). Glycol chitin or glycol chitosan were embedded in gels at 0.01% (w/v) and used as a substrate for chitinase and chitosanase activities, respectively, according to Michaud and Asselin (1995). After electrophoresis, gels were incubated for 4 h at 37 °C in sodium acetate buffer, pH 5.0, at a concentration of 50 mM for chitinase and 10 mM for chitosanase activities. For studying the possibility of double activities (chitinase/chitosanase) of the various isoforms, an equivalent mix of both substrates was embedded in the gel at 0.01% (w/v) final concentration. Electrophoreses were run as described above and incubation of the gels in sodium acetate buffer were carried out at both concentrations (50 and 10 mM).

SDS-PAGE separations were carried out under non-reducing conditions. Samples were denatured by 5 min boiling in the denaturing buffer (Trudel and Asselin, 1989). Apparent molecular masses were determined by co-electrophoresis with prestained SDS-PAGE low range molecular weight standards (Bio-Rad). After electrophoresis, renaturation of chitosanase activities was carried out by a 20 min wash in 200 ml of 10 mM sodium acetate (pH 5.0) with 1% (v/v) purified Triton X-100, followed by incubation at 37 °C in fresh new buffered Triton X-100 solution.

Isoelectrofocusing (IEF) was carried out on precast polyacrylamide gels (Ampholine PAGplate, Biorad) with a broad pH range (3.5–9.5), and 10 μg of proteins from each root treatment were loaded. Apparent isoelectric points were determined by measuring the pH range in each set of IEF electrophoresis. After separation, proteins were transferred to a 7.5% polyacrylamide overlay gel containing either glycol chitin or glycol chitosan as substrate, and blottings were carried out for 3 h or overnight, respectively.

All electrophoreses were repeated at least three times. Chitinase and chitosanase activities on gels were revealed by fluorescent staining using calcofluor white M2R (0.01%, w/v) in 500 mM TRIS-HCl (pH 8.9) and visualized after destaining under UV (365 nm) light (Trudel and Asselin, 1989). True chitosanase activities were confirmed after Coomassie blue staining (Grenier and Asselin, 1990). Gels were photographed using one orange filter (Polaroid film No. 665) and scanned (HP ScanJet 3c). All figures show results corresponding to 6-week-old plants, except for the time-course experiment, for which results from 6, 8, 10, and 12-week-old plants are included. All chemicals used for electrophoresis were from Bio-Rad (Prat de Llobregat, Barcelona, Spain). Other products were from Sigma Chemical Co. (Alcobendas, Madrid, Spain).

Results

Plant growth and fungal colonization

Neither *G. mosseae* nor *G. intraradices* showed a significant effect on plant growth at any of the harvest times under our experimental conditions. On the contrary, the pathogen *P. parasitica* significantly reduced shoot and root growth and caused visible symptoms of necrosis in the root system of the inoculated plants. The presence of *P. parasitica* in roots inoculated with the pathogen was corroborated by ELISA since absorbance values of 1.94 were obtained in extracts of *P. parasitica*-infected roots, while control or mycorrhizal plants showed absorbance values around 0.1, which correspond to the test background value. Concerning mycorrhizal colonization, well-established symbiosis could be observed after 4 weeks of growth, since about 40% of the root system appeared

colonized by the AM fungus *G. mosseae* and about 45% by *G. intraradices*. This percentage increased with time and reached over 65% after 8 weeks for both fungi. Tomato plants inoculated *in vitro* by *Gi. rosea* showed a high level of root colonization by the AM fungus (78%).

Detection of chitinase isoforms

Crude extracts from tomato roots corresponding to both *in vivo* and *in vitro* experiments were submitted to electrophoresis under native conditions to separate acidic or neutral proteins (Fig. 1). In non-mycorrhizal roots, whether the plants were grown axenically or not, three main bands (chit1, chit2 and chit3) were detected, corresponding to constitutively expressed chitinase isoforms (Fig. 1, lanes C). However, chit3 showed a fainter signal in tomato grown *in vitro*. A few other faint bands were also observed. As previously reported (Pozo *et al.*, 1996), a major additional broad band was observed in *G. mosseae*-colonized tomato roots (Fig. 1, lane Gm, arrow). An equivalent banding pattern was also detected in *G. intraradices*-colonized roots (data not shown), as well as in roots monoxenically colonized by *Gi. rosea* (Fig. 1, lane Gir).

In order to characterize the mycorrhiza-related isoform further, protein extracts from non-mycorrhizal, *G. mosseae*- or *G. intraradices*-colonized and *P. parasitica*-infected roots from the *in vivo* experiment were analysed by isoelectric focusing. No obvious qualitative differences in basic chitinase activities occurred between the different root extracts (Fig. 2), although stronger activities were usually detected in *P. parasitica*-infected plants. However, two additional closely-migrating bands, with apparent

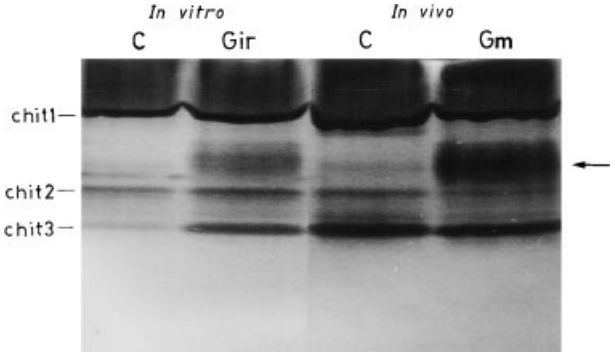

Fig. 1. Acidic chitinase activities after separation of proteins under native conditions at pH 8.9 by the Davis system in 15% (w/v) polyacrylamide gels containing 0.01% (w/v) glycol chitin as substrate. Root extracts (10 μg of proteins/sample) from non-mycorrhizal controls (C) and *Glomus mosseae*-colonized (Gm) tomato plants from the container (*in vivo*) experiment were compared with those from the tomato plants grown *in vitro* either as non-mycorrhizal control (C) or colonized by *Gigaspora rosea* (Gir). Gels were stained with Calcofluor white M2R, visualized and photographed under UV light according to Trudel and Asselin (1989). Constitutive chitinase isoforms are labeled as chit1, chit2 and chit3, and the mycorrhiza-related isoform is indicated by an arrow.

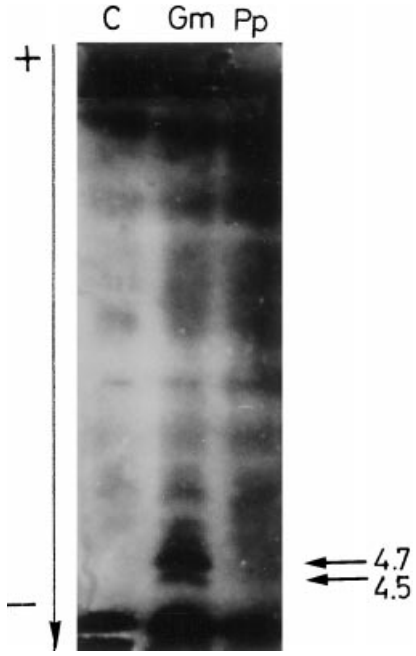

Fig. 2. Chitinase isoenzyme patterns on a 7.5% polyacrylamide overlay gel containing glycol chitin (0.01%, w/v) after IEF in broad pH range (3.5–9.5) precasted gels. Root extracts (10 μg of proteins per sample) from non-mycorrhizal control (C), *G. mosseae*-colonized (Gm) and *P. parasitica*-infected (Pp) plants were electrophoresed. Gels were stained with Calcofluor white M2R. The apparent isoelectric point (pI) of the additional isoforms is indicated.

isoelectric points of 4.5 and 4.7, appeared in extracts from plants colonized with either *G. mosseae* (Fig. 2, lane Gm) or *G. intraradices* (data not shown). These bands were not detected in controls nor in *Phytophthora*-infected roots (Fig. 2, lanes C and Pp).

Detection of chitosanase activities

Chitosanase activities were analysed in tomato root extracts using various electrophoretic systems. Firstly chitosanase expression was studied under native conditions in extracts from roots inoculated with the symbiotic fungi *G. mosseae* or *G. intraradices*, or with *P. parasitica* (Fig. 3). No signal corresponding to chitosanase activity was found in control roots (Fig. 3, lane C), but five main bands with chitosanase activity (chtn1 to chtn5) were observed in mycorrhizal plants (Fig. 3, lanes Gm and Gi). The banding pattern of extracts from plants colonized by either *G. mosseae* (Fig. 3, lane Gm) or *G. intraradices* (Fig. 3, lane Gi) was similar except for some slight differences in the intensity of the bands. In extracts of *P. parasitica*-infected roots, no chitosanase activity was observed (Fig. 3, lane Pp).

Chitosanases are known to be highly sensitive to reducing agents (El Ouakfaoui and Asselin, 1992*a*). Thus, root extracts from *in vitro* and *in vivo* plants were analysed by SDS-PAGE after denaturation without reducing agents. When the plants were grown in containers (*in*

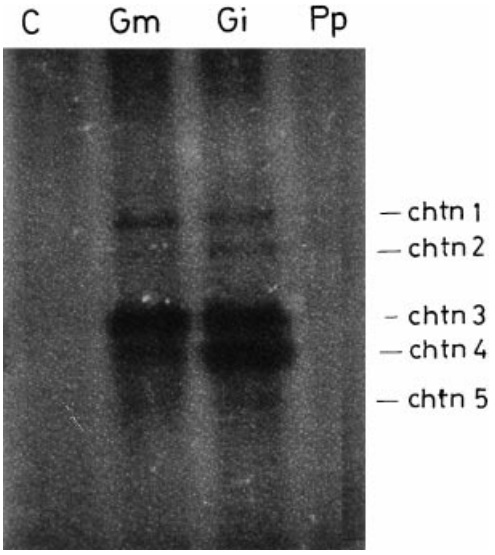

Fig. 3. Acidic chitosanase activities after separation of proteins under native conditions by the Davis system in 15% (w/v) polyacrylamide gels containing 0.01% (w/v) glycol chitosan as substrate. Root extracts (10 μg of proteins per sample) from non-mycorrhizal controls (C), *Glomus mosseae* (Gm) and *Glomus intraradices* (Gi)-colonized, and from *P. parasitica*-infected (Pp) tomato plants were tested. Gels were stained with Calcofluor white M2R, visualized and photographed under UV light according to Trudel and Asselin (1989). Main chitosanase activities are labelled chtn1 to chtn 5.

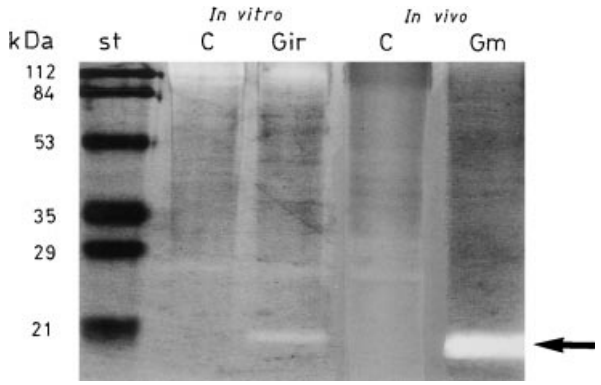

Fig. 4. Chitosanase activities after denaturing polyacrylamide (15% w/v) gel electrophoresis (SDS-PAGE) under non-reducing conditions. Root extracts (10 μg of proteins per sample) from non-mycorrhizal control (C) and *Glomus mosseae*-colonized (Gm) tomato plants from the container (*in vivo*) experiments were compared to those from tomato plants grown *in vitro* either as non-mycorrhizal controls (C) or colonized by *Gigaspora rosea* (Gir). Before electrophoresis, samples were boiled in denaturing buffer without reducing agent; renaturation was done following Trudel and Asselin (1989) during 3 h. 10 μl of total prestained molecular mass markers (st) (low molecular weight Kit from Bio-Rad) were loaded in each gel, and their molecular masses are indicated on the left. Chitosanase activity is indicated by an arrow.

vivo) no signal was detected in control tomato roots (Fig. 4, lane C, *in vivo*). However, a strong signal at approximately 20 kDa was observed in *G. mosseae*-colonized roots (Fig. 4, lane Gm). This strong signal was resolved in some gels as two bands with very close estimated molecular masses (data not shown). They were confirmed as true chitosanases by staining the substrate with Coomasie blue, and a faint band, at the same level of migration to the one observed in *G. mosseae*-colonized tomato from the *in vivo* experiment, was also detected in mycorrhizal plants grown monoxenically (Fig. 4, lane Gir). This band was not present in non-mycorrhizal roots growing axenically. A signal appeared in the upper part of the gel in roots grown *in vitro* (Fig. 4, lanes C and Gir) that was not detected in controls nor in *G. mosseae*-colonized roots from the container experiment.

It was possible to detect chitosanase activity by IEF only after overnight incubation with the substrate. No signal was detected in control nor in pathogen-infected root extracts (Fig. 5, lanes C and Pp), while a diffuse signal, located mainly along the acidic range of the gel and with a maximum activity at around 6.5, was detected in extracts of *G. mosseae*-colonized plants (Fig. 5, lane Gm). These activities, although not resolved as well-defined bands, were confirmed as chitosanases after Coomassie blue staining.

Time-course enzymatic expression

Changes along time in the expression of both chitinases and chitosanases were studied under native conditions by the Davis electrophoretic system during a time-course experiment, using 4, 6, 8, 10, and 12 week-old plants. No

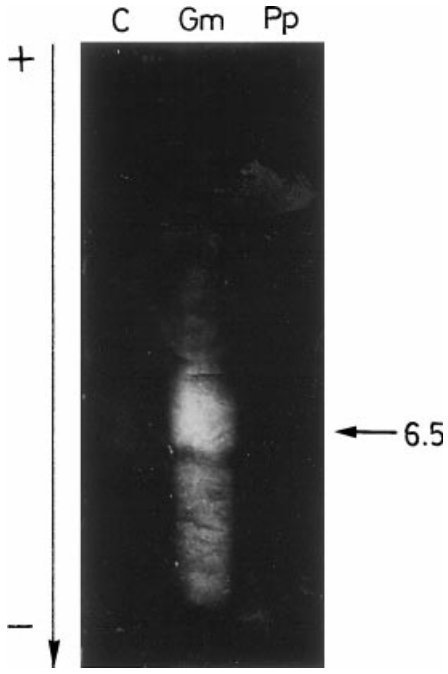

Fig. 5. Chitosanase isoenzyme patterns on a 7.5% polyacrylamide overlay gel containing glycol chitosan 0.01% (w/v) after IEF in broad pH range (3.5–9.5) precasted gels. Root extracts (10 μg of proteins per sample) from non-mycorrhizal control (C), *G. mosseae*-colonized (Gm) and *P. parasitica*-infected (Pp) plants were loaded. Gel was stained with Coomasie blue. The apparent isoelectric point of the main lytic zone is indicated.

significant difference was observed in the chitinase banding pattern either in non-mycorrhizal or in mycorrhizal plants, except for the intensity of the mycorrhiza-related isoform, which seemed to decrease with time (data not shown).

Concerning chitosanases, several differences were observed among the successive plant developmental stages, except for 4 and 6-week-old plants, which showed similar banding patterns. These changes are recorded in Fig. 6. In control roots no chitosanase activity was detected at 4 or 6 weeks (Fig. 6, 6 w, lane C). After 8 weeks of growth, a faint band appeared in the upper part of the gel, and its intensity increased after 10 weeks (Fig. 6, 8 w, 10 w, lanes C). At 12 weeks, another band was also detected in the upper part of the gel (Fig. 6, 12 w, lane C). Five main chitosanase activities were detected in extracts from *G. mosseae*-colonized roots at 4 and 6 weeks of growth (Fig. 6, 6 w, lane Gm). After 8 weeks of growth, the two bands with lower relative mobility (RM) (chtn1 and chtn2) and the one with higher RM (chtn5) were detected only as very faint bands (Fig. 6, 8 w, lane Gm). Two weeks later, these isoforms were not detected, and the activity of the two others isoforms (chtn3 and chtn4) also decreased (Fig. 6, 10 w, lane Gm). After 12 weeks, the mycorrhiza-related chitosanase isoforms were not detected, but a slight signal was observed in the upper part of the gel, at the same level than in control plants but with lower intensity (Fig. 6, l2 w, lane Gm).

Dual chitinase/chitosanase activity

In order to determine if some chitinase isoforms could also degrade chitosan, the detection procedure was modified by embedding the two substrates (glycol chitin and glycol chitosan) simultaneously in the gel matrix. Since *P. parasitica*-infected plants did not show chitosanase activities and no significant difference was found between the isoform pattern of *G. mosseae*- and *G. intraradices*-colonized plants, the focus was on the analysis of root extracts from non-mycorrhizal control plants and plants colonized with *G. mosseae*.

After electrophoresis, gels were incubated in sodium acetate buffer either at 50 mM, as usual for chitinase activity (Fig. 7A, B), or at 10 mM, as for chitosanases (Fig. 7C, D). Gels were firstly stained with Calcofluor white M2R (Fig. 7A, C), a fluorochrome which binds both glycol chitin and glycol chitosan. Lytic zones corresponding to chitinase and chitosanase activities appeared as dark (non-fluorescent) bands against the UV fluorescent background of the intact mixture of substrates. After incubation in 50 mM sodium acetate buffer, four isoforms (Fig. 7A, lane C) were observed in control roots, three of which having similar RM to the chitinase isoforms already detected in control roots (Fig.1, lanes C, chit1, chit2 and chit3). In root extracts from mycorrhizal plants (Fig. 7A, lane Gm), an increase of the total lytic activity was observed. The activity of the chit2 isoform was strongly enhanced, and three new bands, absent from control roots, were detected (Fig. 7A, lane Gm, arrows on the right). When gels were incubated in 10 mM sodium acetate buffer, only three constitutive isoforms were

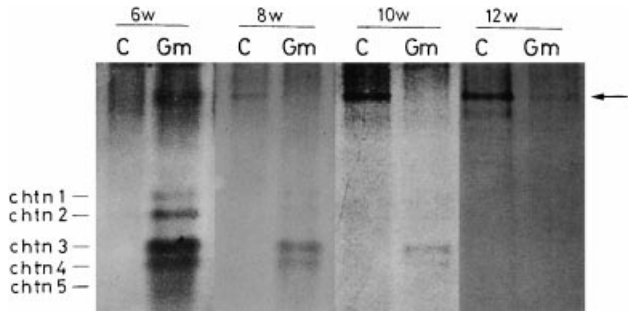

Fig. 6. Time-course study of the chitosanase isoenzyme pattern after native electrophoresis by the Davis system in 15% polyacrylamide gels. Root extracts (10 μg of proteins per sample) from non-mycorrhizal control (C) and *G. mosseae*-colonized (Gm) plants were loaded. Main chitosanase isoforms are indicated on the left, and the age-related isoform is marked with an arrow.

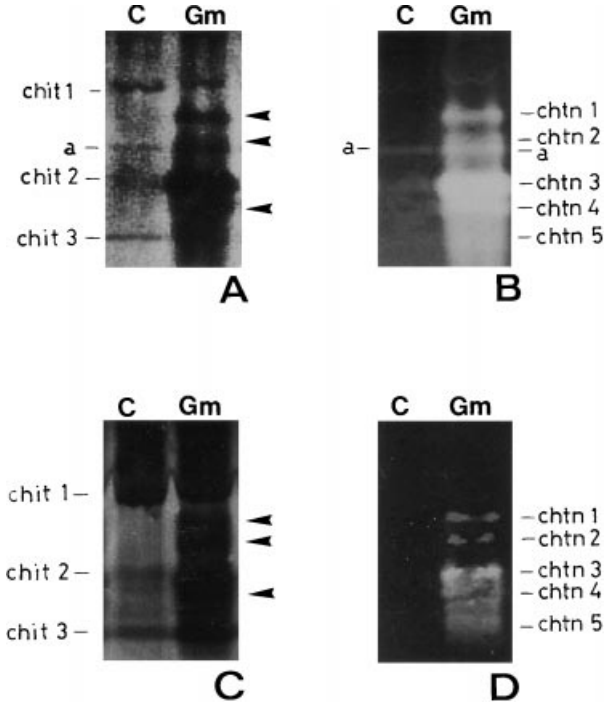

Fig. 7. Isoenzyme pattern after separation by native electrophoresis following the Davis system in 15% polyacrylamide gels containing glycol chitin and glycol chitosan as substrates. Root extracts (10 μg of proteins per sample) of non-mycorrhizal controls (C) and *G. mosseae*-colonized (Gm) plants after 6, 8, 10, and 12 weeks of growth were loaded in each gel. Gels in pannels A and B were incubated in 50 mM sodium acetate buffer, and gels in C and D in the same buffer but at 10 mM. Gels in A and C were stained with Calcofluor MR2, and gels in B and D with Coomasie blue. Main chitinase and chitosanase activities are indicated.

detected in control roots (Fig. 7C, lane C), corresponding apparently to chit1, chit2 and chit3 of Fig. 1 and Fig. 7A. In mycorrhizal roots the activity of the two constitutive isoforms with higher mobility (chit2 and chit3) was also enhanced and three additional bands, not present in control roots, were detected (Fig. 7C, lane Gm, arrows on the right). Two of these isoforms, detected in mycorrhizal roots whatever the incubation process (Fig. 7A, C, lanes Gm), showed a similar RM to the strong signal previously reported as mycorrhiza-related chitinase isoform (Fig. 1, lanes Gm and Gir, arrow on the right). Gels were then stained with Coomasie blue (Fig. 7B, D), to confirm true chitosanase activities. Coomasie blue stains intact glycol chitosan intensely, while lytic zones remain unstained (white bands against the blue background). A positive signal was detected in control roots after incubation in 50 mM sodium acetate buffer (Fig. 7B, lane C, labeled a), while no signal appeared in the same extract after incubation in 10 mM buffer (Fig. 7D, lane C). In *G. mosseae*-colonized plants, besides the isoform observed in controls (Fig. 7B, lane Gm, labeled a), five additional bands were detected after incubation in 50 mM (Fig. 7B, lane Gm, chtn1 to chtn5). These seem to correspond to the three new bands already detected (Fig. 7A, arrows on the right) and the constitutive chitinase isoforms chit2 and chit3. The isoform showing chitosanase activity in either control or mycorrhizal roots (Fig. 7B, a) was not detected after incubation in 10 mM sodium acetate buffer. However, the five bands detected in *G. mosseae*-colonized roots after incubation at 10 mM (Fig. 7D, lane Gm, chtn1 to chtn5) seemed to correspond to those detected after incubation in 50 mM buffer (Fig. 7B, lane Gm). These isoforms seem to be those identified as chtn1 to chtn5 in Fig. 3.

Electrophoresis under denaturing conditions in gels containing both substrates (Fig. 8) revealed four bands in *G. mosseae*-colonized plants after Calcofluor staining (Fig. 8A, lane Gm, arrows on the left), showing MW about 34, 28, 20, and 19 kDa, while only one band was detected in control roots with an apparent molecular mass of 28 kDa (Fig. 8A, lane C). According to their MW the bands of 34 and 28 kDa correspond to the chitinase isoforms previously described in tomato roots (Pozo *et al.*, 1996), while the two bands with lower apparent MW (20 and 19 kDa) correspond to chitosanases, as shown in the present work (Fig. 4, lane Gm). When gels were stained with Coomassie blue (Fig. 8B) the bands at 28, 20 and 19 kDa were revealed as chitosanases in *G. mosseae*-colonized plants (Fig. 8B, lane Gm, arrows on the right). The band at 34 kDa was not detected after Coomasie staining, thus, this chitinase isoform does not appear to display chitosanase activity. No clear signal was observed in control roots (Fig. 8B, lane C).

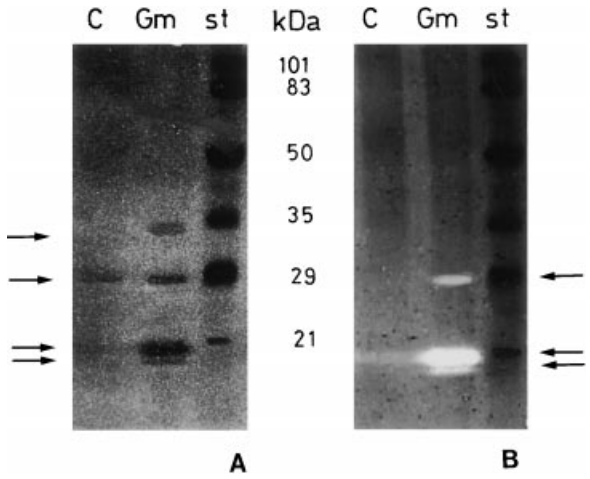

Fig. 8. Chitinase/chitosanase activities after denaturing polyacrylamide (15%, w/v) gel electrophoresis (SDS-PAGE) under non-reducing conditions in gels containing glycol chitin and glycol chitosan as substrates. Root extracts (10 μg of proteins per sample) from non-mycorrhizal control (C) and *Glomus mosseae*-colonized (Gm) tomato plants were tested. Before electrophoresis, samples were boiled in denaturing buffer without reducing agent; renaturation was done in 50 mM sodium acetate and Triton X-100 during 3 h. 10 μl of total prestained molecular mass markers (st) (low molecular weight Kit from Bio-Rad) were loaded in each gel, and their molecular masses are indicated. Lytic activities are indicated by arrows. Gel in panel A was stained with Calcofluor MR2 and gel in panel B with Coomassie blue.

Discussion

Chitinases and β-1,3-glucanases are synergistically induced during attack by fungal pathogens and by fungal elicitors. Their induction is generally considered to be part of a non-specific defence response initiated in plants after pathogen attack, but also a consequence of various physical, chemical and environmental stresses (Sahai and Manocha, 1993). However, the induction of new root acidic chitinase isoforms during AM symbiosis appears to be a specific response, since differential induction of chitinase isoforms after symbiotic or pathogenic fungal interactions has been reported in various plants (Dumas-Gaudot *et al.*, 1992*a*, 1996; Dassi *et al.*, 1996). This was later confirmed in tomato roots during symbiosis with *G. mosseae* or after *Phytophthora parasitica* attack (Pozo *et al.*, 1996). In the present work, a similar induction of specific acidic chitinase isoforms in tomato roots has been shown to be a consequence of interactions with a different AM fungal species (*G. intraradices*), and even with a species belonging to a different genus (*Gigaspora rosea*). Moreover, the use of monoxenically AM-colonized plants rules out the possibility that other microorganisms accompanying the mycorrhizal inoculum play a role in these changes in enzymatic activities. These data bring further evidence for the specificity of the mycorrhiza-related chitinase isoforms in tomato. Isoelectrofocusing allowed a more accurate characterization of these isoforms. Whereas in the Davis system they were detected as a

strong single signal, after IEF it was concluded that there were two isoforms with close isoelectric points (estimated as 4.5 and 4.7).

Tomato root chitosanases were studied during interactions with mycorrhizal or pathogenic fungi for the first time. Under native conditions, no chitosanase isoform was detected in control roots of young and non-stressed tomato plants. This is in agreement with previous data on seeds, leaves or fruits (El Ouakfaoui and Asselin, 1992*a*). In contrast, this study showed the induction of several isoforms in tomato roots upon colonization by either *G. mosseae* or *G. intraradices*. This induction also appears to be a symbiosis-specific response and not part of unspecific defence mechanisms, since *P. parasitica* infection did not induce any chitosanase activity. To our knowledge, there is only one previous report of induction of chitosanase activities after mycorrhizal colonization (Dumas-Gaudot *et al*., 1992*b*).

The main chitosanase activity determined by SDS-PAGE corresponded to an apparent MW of 20 kDa, and a minor one of very close MW, but with lower activity, was also detected. These data are in agreement with the previously described range for chitosanases, of between 10 and 23 kDa (El Ouakfaoui and Asselin, 1992*a*). The induction of chitosanase activity in tomato roots colonized *in vitro* with *Gi. rosea* was also visualized, although with lower intensity than in mycorrhizal plants grown in open containers. When root proteins were submitted to IEF, a chitosanase signal was only detected in mycorrhizal plants. Although no well-defined bands were observed, the general activity appeared in the acidic part of the gel, with a higher signal at a pH of about 6.5.

The induction of similar mycorrhiza-related chitinase and chitosanase isoforms by different species and genera of AM fungi in the same plant (this work and Dumas-Gaudot *et al*., 1992*a*) points to a plant origin for these activities. Other evidence supporting this hypothesis are the induction by the same fungus of isoforms with different MW depending on the plant species (Dumas-Gaudot *et al*., 1992*b*), and the absence of chitinase activity in a mixture of germinated spores and mycelium of *G. mosseae* (Dumas-Gaudot *et al*., 1994). In spite of this evidence, it cannot be discarded that the novel isoforms were produced by the mycorrhizal fungi as host-inducible enzymes.

During the time-course experiment, the mycorrhiza-related chitinase isoforms tended to decrease in activity with plant age. A progressive decrease of chitosanase activities with time has also been observed in mycorrhizal plants, and these isoforms disappeared at the later stages of plant development. El Ouakfaoui and Asselin (1992*b*) have reported that the intensity of the expression of different chitosanase isoforms change, and, in general, slightly decrease with ageing. In this study, however, some other isoforms appeared in both non-mycorrhizal and mycorrhizal plants at late stages, but these isoforms appeared later in mycorrhizal plants than in non-mycorrhizal controls. They could be related to stress conditions. In extracts of tomato roots grown *in vitro*, a signal corresponding to chitosanase activity was detected in the upper part of the gel, regardless of whether the plantlets were inoculated with *Gi. rosea* or not. These signals could be interpreted as a stress response to the *in vitro* growth conditions. The chitosanase isoenzyme pattern appeared to be complex and varied highly depending on the environmental conditions.

Some plant chitinases are able to degrade substrates other than chitin, such as peptidoglycan and chitosan (Mayer *et al*., 1996). It is noteworthy that eukaryotic and prokaryotic chitinases, chitosanases and lysozymes, although sharing no significant amino-acid similarities, have a structurally invariant core consisting of two helices and a three-stranded beta-sheet which form the substrate-binding and catalytic cleft (Monzingo *et al*., 1996). Pegg and Young (1982) purified an endochitinase from tomato stems. It was characterized as a monomeric enzyme able to cleave internal bonds of chitosan, with an isoelectric point of 8.5 and a MW of between 27 and 31 kDa. Although several reports point out hydrolases with both chitinase and chitosanase activities in bacteria and *Citrus* cells (Watanabe *et al*., 1992; Fukamizo *et al*., 1992, 1994; Osswald *et al*., 1992, 1993, 1994; Mayer *et al*., 1995, 1996), this is, as far as is known, the first report of double activities demonstrated by a gel electrophoresis strategy. In the present study it has been shown that in mycorrhizal plants some tomato root chitinase isoforms display chitosanase activity. In non-mycorrhizal plants, only one of the constitutive chitinase isoforms, under specific incubation conditions (50 mM sodium acetate buffer), presented some chitosanase activity. In contrast, in mycorrhizal plants some constitutive chitinase isoforms (chit2 and chit3), together with the two additional mycorrhiza-related chitinase isoforms were able to degrade chitosan (chtn1, chtn2, chtn3, and chtn5). A different mycorrhiza-related isoform showed exclusively chitosanase activity (chtn4), since it was not detected in native gels containing only glycol chitin as substrate. Although the possibility cannot be ruled out that some chitosanase and chitinase isoforms co-migrate with the same RM, it seems unlikely that this situation has occurred for the different bands revealed here. On the other hand, the degree of acetylation of chitosan is important in studies on enzymes with double chitinase/chitosanase activity. Several chitinases isolated from callus cultures of *Citrus sinensis* showed different substrate specificities when using chitosans of varying chain length and degrees of acetylation (Mayer *et al*., 1996). Osswald *et al*. (1992) also found large differences in the activity detected using different chitosan preparations. Later on these authors reported the purification of eight chitinase isoforms from *Citrus* callus tissue of which only four were able to degrade chitosan.

The chitosanase activity of these chitinase-chitosanase enzymes was optimal with 18–20% acetylated chitosan. It decreased with decreasing acetylation, but they were still active on completely deacetylated chitosan. They did not rule out the possibility that some of the isoforms are the same, varying only in a few amino acids as a result of post-transcriptional modifications (Osswald *et al*., 1994). In the present study, 95% acetylated glycol chitin, (Sannan *et al*., 1976; Grenier and Asselin, 1990) and 94% deacetylated glycol chitosan (El Ouakfaoui and Asselin, 1992*a*) were used as substrates. Further research using chitosans with different degrees of acetylation would be necessary to determine the substrate specificity of the different tomato root chitinase isoforms.

Dual chitinase/chitosanase activities were also studied under denaturing conditions. One lytic band of 28 kDa, which did not show chitosanase activity, was detected in control roots. In *G. mosseae*-colonized plants, four bands of 34, 28, 20, and 19 kDa were detected. The bands at 34 and 28 kDa, according to their MW, correspond to the chitinase isoforms previously described in tomato roots (Pozo *et al*., 1996). The two lower bands correspond to the chitosanases revealed in this study in gels containing only glycol chitosan as substrate. Their activity as chitosanases was confirmed after Coomasie blue staining. Moreover, the band at 28 kDa was also shown to display chitosanase activity. To summarize, under denaturing conditions a 34 kDa chitinase without chitosanase activity, a 28 kDa chitinase which was capable of degrading chitosan, and two closely-migrating chitosanases with apparent MW of 20 and 19 kDa were found in mycorrhizal root extracts.

In a biological system, from the standpoint of energy conservation, it would be more efficient to have multifunctional enzymes (Osswald *et al*., 1994). The broad substrate hydrolytic ability of chitinases-chitosanases towards differently acetylated chitosans (Fukamizo *et al*., 1992, 1994) may enable the plant to resist a greater number of chitin- and chitosan-containing pathogens better (Mayer *et al*., 1995). In fact, chitosans isolated from fungal sources are partially *N*-acetylated (Wessels and Sietsma, 1981). The antifungal properties of chitosanases have been demonstrated *in vitro*. Purified chitosanase from *Streptomyces* caused growth inhibition of different fungi including an Oomycete (*Pythium*), and the enzyme was active when expressed in transgenic tobacco (El Ouakfaoui *et al*., 1995). Some chitosanases, including a basic one from the intercellular fluid of chemically stressed tomato leaves, were able to lyse spores of *Fusarium oxysporum*, *Verticillium albo-atrum* and *Ophiostoma ulmi* embedded in polyacrylamide gels (Grenier and Asselin, 1990). Thus, chitosanases induced upon mycorrhizal colonization could play a role in bioprotection by working in concert with chitinases and β-1,3-glucanases against invading root pathogens. This could be exerted by acting either directly through their lytic activity, or indirectly, by releasing oligosaccharides from the pathogen cell wall that could elicit host defence reactions.

On the other hand, chitinase and chitosanase activities are very likely to be a key point in the establishment and functioning of the AM symbiosis. Although their precise function in symbiotic interactions is still unclear, stimulation of plant chitinase activities has been reported in several root symbioses, such as soybean nodules (Staehelin *et al*., 1992), ectomycorrhizas (Albrecht *et al*., 1993) and arbuscular mycorrhizas (Spanu *et al*., 1989; Dumas-Gaudot *et al*., 1992*a*, *b*, 1994; Volpin *et al*., 1994; Pozo *et al*., 1996). Particularly, Volpin *et al*. (1994) found that the accumulation of mRNAs encoding chitinases was shown in cells containing arbuscules (Blee and Anderson, 1996), which suggests a role for these enzymes in the regulation of the symbiosis. Concerning *Rhizobium* symbiosis, Nod factors have been shown to be substrates for plant chitinases (Schultze *et al*., 1993), and structural parameters determining host specificity (length of the oligosaccharide chain, acylation of the non-reducing end and sulphation at the reducing end) influence the stability of the molecule against degradation by chitinases (Staehelin *et al*., 1994*b*). Consequently, it has been postulated that chitinases, among others, may be involved in the recognition of the rhizobial nodulation signals and thus, in the self-regulation of the nodulation process (Staehelin *et al*., 1994*b*; Mellor and Collinge, 1995). Interestingly, highly purified Nod factors increased the degree of mycorrhizal colonization in soybean (Xie *et al*., 1995). Although tomato is a non-host for nodulating rhizobia, it has been demonstrated in tomato cells that there is a sensitive perception system for chito-oligosaccharides which can recognize Nod factors (Felix *et al*., 1993; Staehelin *et al*., 1994*a*). It is tempting to speculate that mycorrhizal symbiosis could have exploited this system for symbiotic signal perception, and modified chitin or chitosan oligomers released from the AM fungal cell walls might be good candidates as signalling compounds during the infection process in arbuscular mycorrhizas. The modulation of the chitinase substrate specificity could be a key point in the exchange of signals between symbionts, and, finally, in the recognition processes.

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