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Mycorrhiza-Related Chitinase and Chitosanase Activity Isoforms in *Medicago truncatula* Gaertn.

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Abstract

After inoculation with the arbuscular mycorrhizal fungus *Glomus mosseae*, additional chitinase and chitosanase isoforms were detected in *Medicago truncatula* roots. Chitinase isoforms had isoelectric points between 5.3 and 5.8 and apparent molecular weights of 30 to 40 kDa. Western blotting demonstrated the cross-reactivity of the 30 kDa isoforms with a winged bean class I chitinase and a class III chitinase from chickpea, while the 40 kDa isoform only cross-reacted with the class III chitinase from chickpea. Inoculation with other soil microorganisms showed that these isoforms were strictly induced during the mycorrhizal process. Chitosanase isoforms had apparent molecular weights of 18 to 21 kDa. The three basic isoforms could be detected in nodules isolated from *Sinorhizobium meliloti*-inoculated plants, whereas the two acidic isoforms were strictly induced during mycorrhization. Elicitation with various chitooligosaccharides neither induced any of the mycorrhiza-related chitinase and chitosanase isoforms, nor had an effect on mycorrhizal infection, after subsequent transplantation of elicited plants into pots containing *G. mosseae* inoculum. A hypothetical role of chitinases and chitosanases in the formation and/or degradation of arbuscules, is discussed.

Keywords: Arbuscular mycorrhizal symbiosis, *Medicago truncatula*, *Glomus mosseae*, elicitor, chitinase, chitosanase

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1. Introduction

Plants are exposed in nature to a wide range of microorganisms. While pathogenic microbes establish relationships which are detrimental to the plant, others like mycorrhizal fungi are beneficial to their hosts. Arbuscular mycorrhizal fungi, which are members of the class Zygomycetes, order Glomales, are obligate symbionts colonizing more than 80% of terrestrial plants (Harley and Smith, 1983). The colonization process is characterized by intercellular and intracellular hyphal growth in the root cortical tissue with differentiation of terminal intracellular branched haustoria called arbuscules. Through these specialized structures, a bi-directional exchange of nutrients occurs between the fungal hyphae and the plant root cells. The plant benefits from a better supply of mineral nutrients, mainly phosphorus, whilst in turn, the fungus receives carbohydrates (Smith and Gianinazzi-Pearson, 1988). Although the process of colonization has been clearly described, the signalling between the two symbionts and the molecular mechanisms underlying the development of the symbiosis are still largely unknown. Several studies have focussed on genes and proteins previously shown to be induced in plant-pathogen interactions, such as phenylalanine ammonia lyase and chalcone synthase, two enzymes of the phenylpropanoid pathway (Lambais and Medhy, 1993; Harrison and Dixon, 1994), as well as the pathogenesis-related proteins of the PR1 group (Gianinazzi-Pearson et al., 1992; Dassi et al., 1996), peroxidase (Franken and Gnädinger, 1994; Spanu and Bonfante-Fasolo, 1988), β-1,3-glucanase, chitinase and chitosanase (Dumas-Gaudot et al., 1992b; Lambais and Medhy, 1996; Pozo et al., 1998; Spanu et al., 1989).

Chitinases (EC 3.2.1.14) catalyse the hydrolysis of chitin, a linear homopolymer of β-1,4 linked N-acetylglucosamine residues, which is a major component of the cell wall of most fungi, including arbuscular mycorrhizal fungi (Wessels and Siestma, 1981). These enzymes are expressed constitutively at low levels but are differentially regulated by numerous biotic and abiotic factors. They are encoded by a small multigenic family and exist as various isoforms differing in their subcellular localization, biochemical characteristics and primary structure (Punja and Zhang, 1993; Graham and Sticklen, 1994). Concerning mycorrhization, some authors reported a transient increase of total chitinase activity early in the development of the symbiosis, probably corresponding to a typical defense reaction (Spanu et al., 1989; Volpin et al., 1994). Others showed the induction of specific mycorrhiza-related isoforms during the late stages of the symbiosis (Dumas-Gaudot et al., 1992a; Slezack et al., 1996). Recently, the expression of mycorrhiza-specific chitinase genes was demonstrated (Salzer et al., 2000; Bonanomi et al., 2001).

Chitosanases (EC 3.2.1.99) act on chitosan, the deacetylated form of chitin.

As for chitinases, several chitosanase isoforms were induced in plants stressed with chemicals or pathogens (Grenier and Asselin, 1990) and some were found to be developmentally regulated (El Ouakfaoui and Asselin, 1992). Chitosan is also a cell wall component of arbuscular mycorrhizal fungi (Bonfante-Fasolo and Grippiolo, 1984) and the occurrence of specific chitosanase activities was reported in different mycorrhizal plants (Dumas-Gaudot et al., 1992b; Pozo et al., 1998; Slezack et al., 2000).

In the present work, we report the induction of chitinase and chitosanase isoforms in the model plant *Medicago truncatula*, in response to colonization by the arbuscular mycorrhizal fungus *Glomus mosseae*. These activity isoforms were characterized by different electrophoretic techniques. To investigate about their induction specificity, plants were inoculated with another mycorrhizal fungus, *Glomus intraradices*, with the symbiotic nitrogen-fixing bacterium *Sinorhizobium meliloti*, or with a pathogenic fungus, *Aphanomyces euteiches*. Control plant roots were treated with various chito-oligosaccharide elicitors including *S. meliloti* Nod factors, chitin, chitosan and N-acetylglucosamine polymers with degrees of polymerization ranging from 1 to 8. Further characterization was achieved by using *M. truncatula* mutants deficient for mycorrhization.

2. Materials and Methods

Plant material and inoculation conditions

Seeds from *Medicago truncatula* Gaertn. cv Jemalong J5 and its isogenic mutant TRV25 (Sagan et al., 1995) were scarified with 85% sulfuric acid (Prolabo, France), surface sterilized with a 12° solution of commercial bleach (Argos, France) and pregerminated for 3 days at 27°C on Petri dishes containing 20 ml of sterile agar (0.7%). The seedlings were then transplanted into 200 ml pots containing a sterile mix of Terragreen (charred montmorillonite clay, Oil Dry US-special type III-R, IMC Imcore) and Epoisses soil (2:1, v/v). Mycorrhizal inoculation was performed by replacing Epoisses soil with a soil-based inoculum containing propagules of either *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) or *Glomus intraradices* Smith & Schenck (BEG 9) [27]. Part of the *G. mosseae* inoculum was dissolved in sterile deionized water and sieved through a filter paper (Filtres Laurent n°1, Prolabo) so as to obtain a suspension containing soil particles and mycorrhizospheric microorganisms, but no fungal spores. This filtrate was used to water a set of pots for a complementary control. Inoculation with *Sinorhizobium meliloti* strain RCR 2011 was realized by watering the planted seedlings with a suspension of bacteria showing an absorbance of 0.5 at 600 nm (1 ml per plant). *Aphanomyces*

euteiches Drechs. strain SRSF 502 was applied on 2-week old plantlets as a zoospore suspension obtained as described by Slezack et al. (1999).

Growth and harvest conditions

Plants were grown in a climatic room under controlled environment (16h photoperiod, 23°C/18°C day/night temperature, 60% relative humidity, 300 µEinstein.m^{-2}.s^{-1} photon flux density) and watered daily either with deionized water or with a modified Long Ashton nutrient solution (Hewitt, 1966) containing one-tenth nitrogen or one-tenth phosphorus for *S. meliloti*- and *G. mosseae*-inoculated plants, respectively (3 times per week). At time of harvest (4 d to 6 wk after inoculation), plants were carefully washed in running tap water, rinsed with deionized water and blotted against paper before weighing aerial parts and roots separately. For some experiments, nodules from *S. meliloti*-inoculated roots were isolated on ice. Nodules as well as whole root systems from control, *G. mosseae*-inoculated and *S. meliloti*-inoculated plants were immediately frozen in liquid nitrogen and stored at –80°C until protein extraction.

Elicitor treatments

To study the induction specifity of chitinase and chitosanase activities, root parts of two-week old control plants were immersed for 24 to 84 hours in *G. mosseae* inoculum filtrate or in solutions containing 10^{-9} M *S. meliloti* Nod factors (kindly provided by Dr. V. Poinsot, Toulouse), 4 mg/ml glycol-chitosan, 1 mg/ml glycol-chitin, 10^{-6} M N-acetylglucosamine, N,NI-acetylchitobiose, N,NI,NII-acetylchitotriose, N,NI,NII,NIII-acetylchitotetraose, N,NI,NII, NIII,NIV-acetylchitopentaose, N,NI,NII,NIII,NIV,NV-acetylchitohexaose, N,NI,NII,NIII,NIV,NV,NVI-acetylchitoheptaose and N,NI,NII,NIII,NIV, NV,NVI,NVII-acetylchitooctaose. For half of the plants, roots were rinsed with sterile deionized water, frozen in liquid nitrogen and stored at –80°C for further protein extraction. The other half was retransplanted into pots containing *G. mosseae* inoculum, grown for 10 days in a climatic room and harvested as described above.

Estimation of root infection

Mycorrhizal colonization was estimated on randomly collected root parts which were cleared and stained with trypan blue (Phillips and Hayman, 1970). The frequency of infection (F%), the percentage of colonized cortex (M%), and the intensity of arbuscule development (A%) were determined according to

Trouvelot et al. (1986). *S. meliloti* inoculation was evaluated by counting nodules. *A. euteiches* infection was estimated by root rot rating scores as described by Rao et al. (1995).

Protein extraction

Frozen samples were ground with liquid nitrogen in an ice-chilled mortar and the resulting powder was resuspended (1 $ml.g^{-1}$ fresh weight) in 100 mM pH 6.8 McIlvaine extracting buffer (McIlvaine, 1921). Crude homogenates were centrifuged at 12,000 g for 1 hour at 4°C and the supernatant fractions containing soluble proteins were collected. Protein content was determined according to the method of Bradford (1976) using BSA as a standard. The extracts were kept frozen at –20°C until further analysis.

Chitinase and chitosanase in-gel activity detection

Samples were subjected to native 15% polyacrylamide gel electrophoresis (PAGE) as described by Davis (1964) for acidic and neutral proteins and by Reisfeld et al. (1962) for basic proteins. Substrates were either directly embedded in the gel matrix or in a 7% polyacrylamide overlay gel. Glycol chitin and glycol chitosan were used respectively for chitinase and chitosanase activity detection, at a concentration of 0.01% (Michaud and Asselin, 1995). Protein extracts were mixed (1:3, v/v) with a solution of 60% sucrose containing 0.04% bromophenol blue (Davis) or methylene blue (Reisfeld) as a tracking dye. After electrophoresis, gels containing the substrates were incubated for 3 hours at 37°C in pH 5.0 sodium acetate buffer, at a concentration of 50 mM for chitinase and 10 mM for chitosanases. When gels did not contain any substrate, they were blotted onto overlay gels and placed at 37°C for 3 hours.

SDS-PAGE separations were achieved in 12 % polyacrylamide gels according to Laemmli (1970) under non-reducing conditions. Gels contained either 0.01% glycol-chitin or glycol-chitosan. Samples were mixed (1:3, v/v) with SDS-sucrose buffer (Trudel and Asselin, 1989) and denatured by boiling for 5 min. Apparent molecular masses were determined by co-electrophoresis with prestained low-range molecular weight standards (Bio-Rad). Electrophoresis was run at 20 mA and renaturation was performed by incubating the gels for 18h at 37°C in sodium acetate buffer with 1% purified Triton X-100.

Isoelectricfocusing (IEF) was carried out on precast 5% polyacrylamide gels (Ampholine PAG Plate pH 3.5–9.5, Amersham Pharmacia Biotech) using a Multiphor II (LKB) system according to the manufacturer's protocol. Apparent isoelectric point was measured by co-electrophoresis with IEF standards (Broad pI kit, Amersham Pharmacia Biotech). After separation, gels were

equilibrated for 5 min in 50 mM sodium acetate buffer (pH 5.0) and overlayed with 7.5% polyacrylamide gels containing 0.02% glycol chitin. The sandwich gels were placed at 37°C for 3h under moist conditions.

Chitinase and chitosanase activities were revealed under UV light (365 nm) after staining the gels for 5 min with the fluorescent dye Calcofluor white M2R (0.01% in Tris-HCl pH 8.9) and destaining with deionized water (Trudel and Asselin, 1989). Chitosanase activities were confirmed after Coomassie blue staining (Grenier and Asselin, 1990).

Western blotting was achieved by transferring proteins onto nitrocellulose membranes (Schleicher and Schuell, 0.45 mm) after separation by SDS-PAGE under non reducing conditions (Towbin et al., 1979). Chitinases were detected by incubating the membranes with polyclonal antibodies directed against a class III chitinase from sugarbeet (Mikkelsen et al., 1992), a class III chitinase from chickpea (Nielsen et al., 1993) and a basic class I chitinase from winged bean (Esaka et al., 1994). After washing, blots were incubated with a secondary antibody coupled to alkaline phosphatase and revealed using α-naphtyl phosphate (Sigma) as a substrate (Burgermeister and Koening, 1984).

Gels and immunoblots were repeated at least twice and were digitalized using a CCD camera (BioPrint, Vilbert Lourma, France).

3. Results

Plant growth and infection parameters

Plant growth as estimated by root and shoot fresh weight was not significantly modified upon mycorrhization with *G. mosseae* or *G. intraradices*. The microscopic study of trypan blue stained *G. mosseae*-inoculated roots revealed that 4 days after inoculation, only appressoria could be seen. Seven days after inoculation, inter- and intra-cellular hyphae as well as arbuscules could be observed sporadically over the root system. Starting from 14 days after inoculation, the maximum infection frequency was reached, and the mycorrhization intensity increased continuously until 42 days after inoculation to reach a value of 71%. Inoculation of *M. truncatula* seedlings with *G. intraradices* led to a lower mycorrhization than that obtained with *G. mosseae*, with about 46% of the root system being colonized 42 days after inoculation.

In *S. meliloti*-inoculated plants, nodules appeared after 14 days of growth, to reach a value of 23 nodules per gram of root fresh weight after 42 days of growth.

A. euteiches significantly reduced shoot and root weight and caused visible symptoms of necrosis on the root system since 5 days after inoculation.

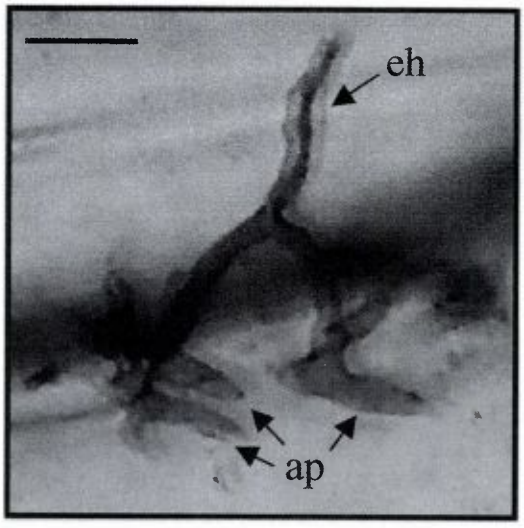

Figure 1. Detail of some appressorial structure formed by *G. mosseae* at the surface of the trypan-blue stained roots of *M. truncatula* mutant line TRV25. eh: extracellular hyphae, ap: appressoria. Scale bar = 100 µm.

Growth of TRV25 mutant plants was similar to the wild-type, and plants were neither colonized by *S. meliloti* nor by *G. mosseae*, although in the latter case, appressoria could be observed at the root surface. Contrary to wild-type roots where a single appressorium was sufficient for the fungus to enter, there were multiple appressoria at the site of a tentative penetration. These appressoria were ramified, often branched out into 2 or more contact structures (Fig. 1). This alteration has also been shown with another mycorrhiza-defective *M. truncatula* mutant (Calantzis et al., 2001).

New chitinase activities in the Medicago truncatula-Glomus mosseae symbiosis

Crude extracts from 42 day old *Medicago truncatula* roots were submitted to Davis native electrophoresis to separate acidic and neutral proteins and revealed for chitinase activities (Fig. 2A). In control roots, two main bands were observed, corresponding to the activities of constitutive chitinase isoforms. In *G. mosseae*-colonized roots, the activity of the upper constitutive isoform was enhanced and above this isoform, a strong additional band (indicated by an arrowhead) was detected.

Native electrophoresis with Reisfeld system to separate basic proteins allowed to detect four constitutive chitinase isoforms but no differences were found between control and *G. mosseae*-inoculated roots (data not shown).

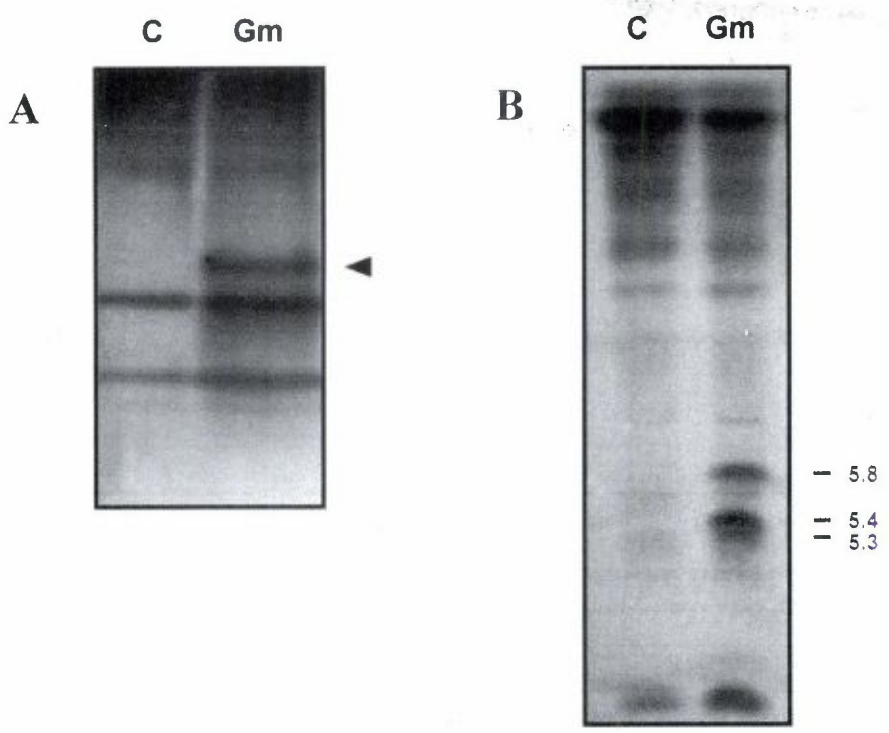

Figure 2. Detection of neutral and acidic chitinase activities after separation of *M. truncatula* root extracts by Davis native polyacrylamide gel electrophoresis (A) and isoelectricfocusing (B). 20 µg of control (C) and *G. mosseae*-inoculated (Gm) root proteins were loaded in each lane. Gels were stained with 0.01% Calcofluor and chitinase activities were visualized under UV light as dark zones corresponding to lytic activities against glycol chitin embedded in the gel matrix (A) or in the overlay gel (B). Numbers on the right correspond to isoelectric points (B).

Isoelectricfocusing (Fig. 2B) confirmed the induction of not only one but several additional acidic chitinase isoforms in *G. mosseae*-inoculated roots, with two major bands showing isoelectric points of 5.8 and 5.4 and one fainter with an isoelectric point of 5.3.

For further characterization, protein extracts were subsequently analysed by SDS-PAGE and Western blotting (Fig. 3). As with IEF, separation of proteins through SDS-PAGE allowed to detect three additional chitinase isoforms with apparent molecular weights between 30 and 40 kDa (Fig. 3A). SDS-PAGE also showed the reinforcement of the activity of one constitutive chitinase isoform behaving with an apparent molecular weight of 34 kDa. Analysis of protein

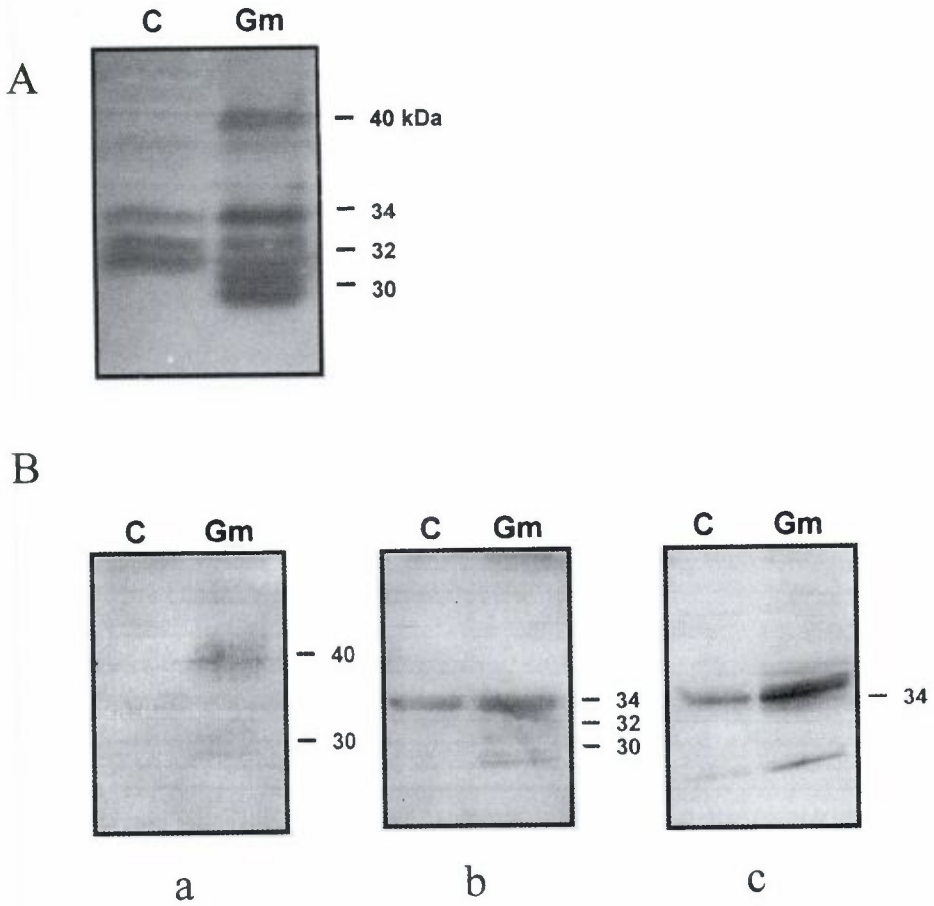

Figure 3. SDS-PAGE (A) and Western blotting (B) of control (C) and *G. mosseae*-inoculated (Gm) root extracts. 20 µg of proteins were loaded in each lane. Numbers on the right indicate the apparent molecular weights of the chitinase isoforms. Antibodies were directed against a chickpea class III chitinase (a), a winged bean class I chitinase (b) or a sugar beet class III chitinase (c).

extracts by Western blotting (Fig. 3B) allowed to discriminate between constitutive and mycorrhiza-induced isoforms. The 40 kDa and 30 kDa mycorrhiza-induced isoforms were immuno-detected in *G. mosseae*-inoculated roots with the chickpea class III antibody (Fig. 3Ba). The 30 kDa isoforms were also detected in mycorrhizal roots with the winged bean class I antibodies (Fig. 3Bb). The 34 kDa constitutive isoform cross-reacted both with the winged bean class I and the sugarbeet class III chitinase antibodies (Figs. 3Bb and 3Bc). The

signal was higher in mycorrhizal roots indicating that the increase in activity observed in SDS-PAGE (Fig. 3A) corresponded to an increase in protein amount.

Specificity of the M. truncatula mycorrhiza-induced chitinase activities

To investigate whether the additional chitinase isoforms observed in the *M. truncatula-G. mosseae* symbiosis are specifically induced or are part of an overall response of the plant to colonization, plantlets were inoculated with several microorganisms such as the nitrogen fixing symbiont *S. meliloti* or the pathogenic fungus *A. euteiches*, as well as with another mycorrhizal fungus, *G. intraradices*. Root protein extracts were subjected to Davis native electrophoresis and isoelectricfocusing (Figs. 4 and 5). In Davis gels (Fig. 4), whereas no difference could be observed between control and *S. meliloti*-inoculated roots, an additional chitinase activity migrating between the two constitutive isoforms (indicated by a star) was detected in isolated nodules, as well as a major additional isoform in the top of the gel. The latter isoform was also present in *A. euteiches*-infected roots (1), where 4 other additional bands (2 to 5) were observed: a first isoform migrated just above the upper constitutive isoform, and the 3 other isoforms were detected in the bottom of the gel, below the lower constitutive isoform. Interestingly, like in *G. mosseae*-inoculated roots, the activity of the upper constitutive isoform was stronger in *A. euteiches*-infected roots, in comparison to control roots. Protein extracts were

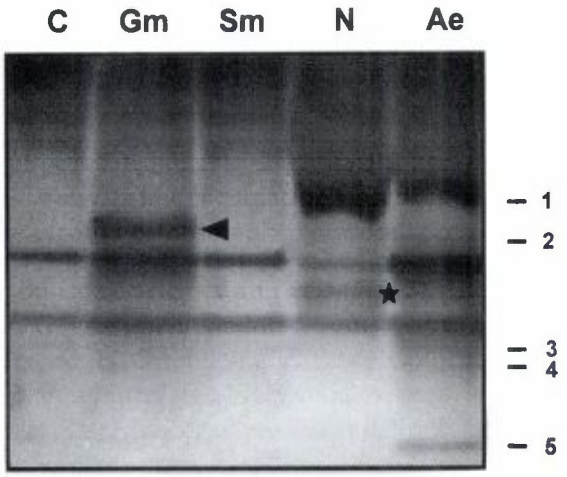

Figure 4. Determination of the specificity of induction of the *M. truncatula* mycorrhiza-induced chitinase activities. Root extracts were separated by Davis native electrophoresis. 20 µg of proteins were loaded in each lane. Samples were from control (C), *G. mosseae* (Gm), *S. meliloti* (Sm), *A. euteiches* (Ae) inoculated plants, and from nodules (N).

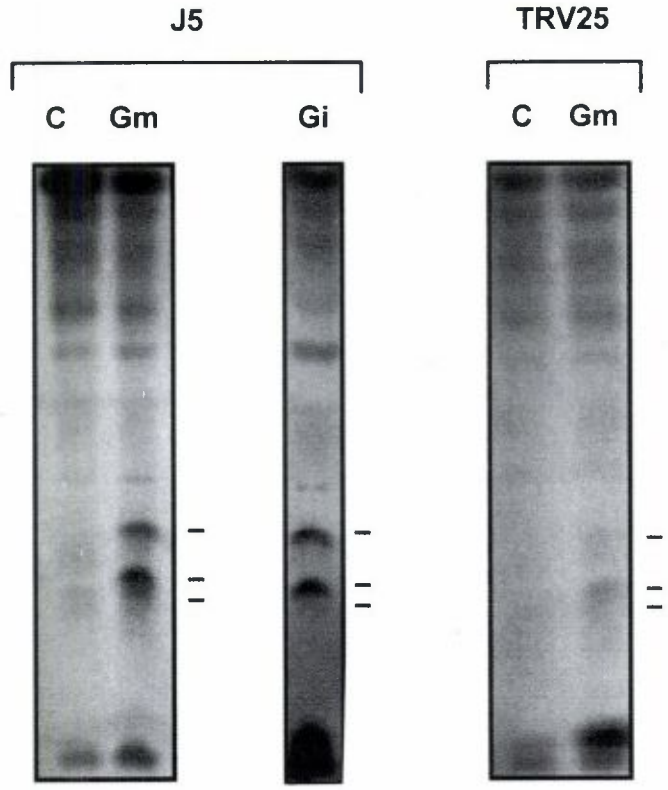

Figure 5. Isoelectricfocusing determination of the specificity of induction of the *M. truncatula* mycorrhiza-induced chitinase isoforms. 20 µg of proteins were loaded in each lane. Samples were from control (C), *G. mosseae* (Gm), *G. intraradices* (Gi) of the wild-type J5 or mutant plants TRV25. The mycorrhiza-related chitinase isoforms are indicated on the right.

also run on isoelectricfocusing gels (Fig. 5). The 3 mycorrhiza-induced isoforms found in *G. mosseae*-inoculated roots were also detected in *G. intraradices*-inoculated roots. Interestingly, these isoforms were also faintly detected in mutant plants inoculated with *G. mosseae*.

To investigate more about the induction of mycorrhiza-induced chitinase isoforms, another experiment was led, where root parts of control *M. truncatula* 2 week-old plantlets were immersed in *G. mosseae* inoculum filtrate as well as in solutions containing *S. meliloti* Nod factors, glycol-chitin, glycol-chitosan and N-acetylglucosamine polymers with degrees of polymerization ranging from 1 to 8. Upon these elicitor treatments, isoelectricfocusing of root extracts

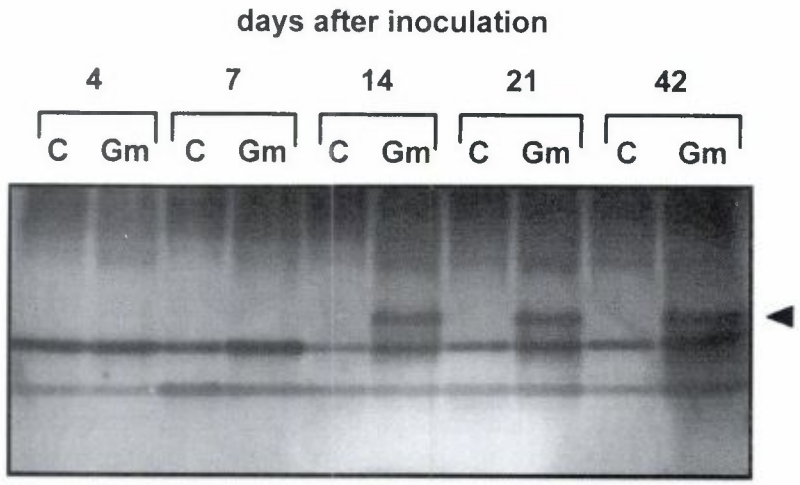

Figure 6. Time-course analysis by Davis electrophoresis of chitinase activities after separation of root extracts from control (C) and *G. mosseae*-inoculated (Gm) *M. truncatula* plants aged 4, 7, 14, 21 and 42 days. 20 µg of proteins were loaded in each lane.

did not show any modification in the chitinase activity pattern after 24, 48 and 84 hours of immersion (data not shown). When plants were retransplanted into pots containing *G. mosseae* inoculum, no differences were observed in the mycorrhizal parameters of the differently treated plants, neither in their chitinase activity pattern as revealed by Davis native electrophoresis (data not shown).

Time-course accumulation pattern of the mycorrhiza-related chitinase isoforms

Time-course analysis of chitinase activities was performed by Davis native electrophoresis and isoelectricfocusing. In Davis system (Fig. 6), the mycorrhiza-related isoform was shown to appear 14 days after inoculation and its activity was stronger 21 and 42 days after inoculation. This was confirmed by isoelectricfocusing with the same pattern of induction for the 3 additional isoforms (data not shown). This increase in activity was parallel to an increase in colonization and especially in arbuscule content as revealed by the mycorrhizal parameters described earlier.

A chitinase isoform is transiently induced during early steps of the symbiosis

Time-course analysis of chitinase activities in isoelectricfocusing gels

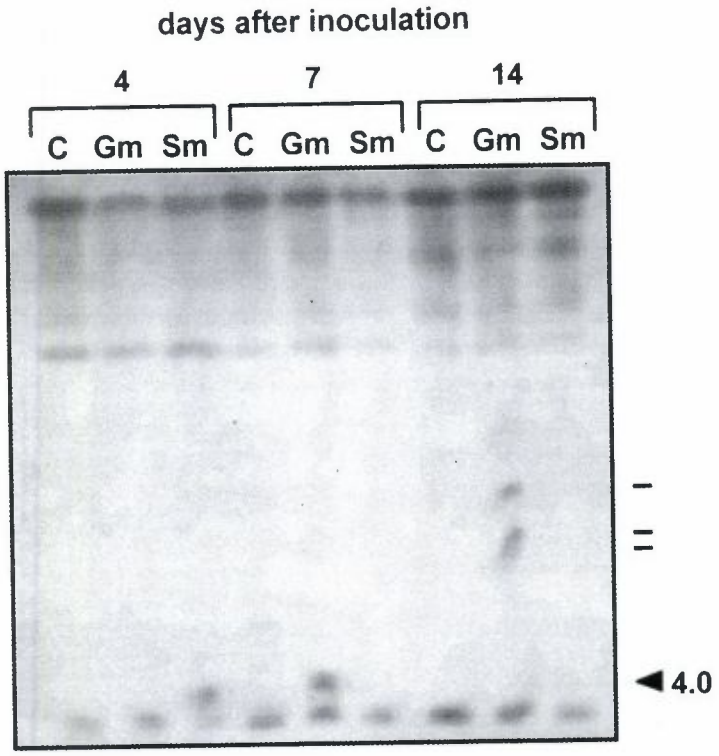

Figure 7. Time-course analysis by isoelectricfocusing of chitinase activities induced during early steps of the symbiosis. 20 µg of proteins from control (C), *G. mosseae*-inoculated (Gm) and *S. meliloti*-inoculated (Sm) *M. truncatula* root extracts were loaded in each lane. The early induced chitinase isoform is indicated by a black arrowhead. The number on the right corresponds to its isoelectric point.

revealed the early and transient induction of a very acidic chitinase isoform with an apparent isoelectric point of 4.0 in 7 day old *G. mosseae*-inoculated roots (Fig. 7). This isoform was not observed 4 days after inoculation and it was no longer detected 14 days after inoculation. Interestingly, the same isoform was observed in 4 day old *S. meliloti*-inoculated roots, and followed a similar pattern, being no longer detected 7 days after inoculation. This isoform was not detected neither in *A. euteiches*-inoculated roots nor in roots treated with the various elicitors as described above.

New chitosanase activities in mycorrhizal M. truncatula

Crude extracts from 42 day old *M. truncatula* roots were separated by SDS-PAGE. After renaturation, chitosanase activities were revealed by Calcofluor

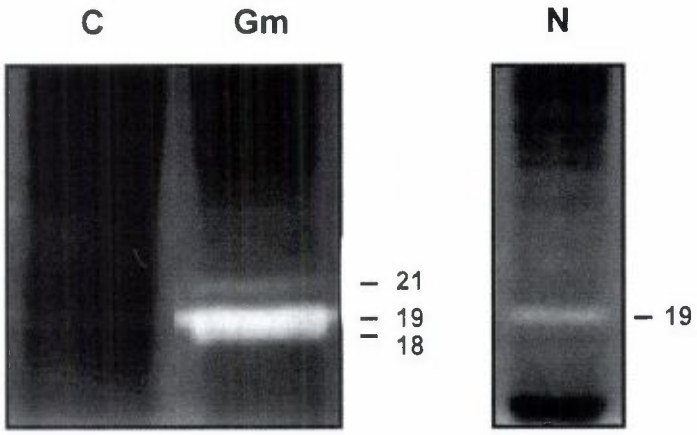

Figure 8. Detection of chitosanase activities after separation by SDS-PAGE of extracts from control (C), *G. mosseae*-inoculated roots (Gm) and nodules (N). 20 µg of proteins were loaded in each lane. After renaturation, chitosanase activities were revealed by Calcofluor and Coomassie blue double-staining, appearing as white bands against a blue background. The numbers on the right correspond to molecular weights in kDa.

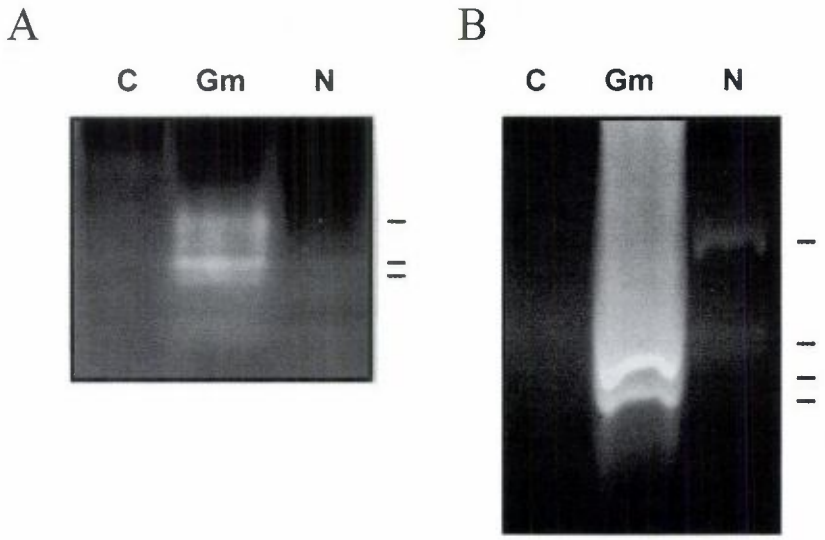

Figure 9. Further characterization of *M. truncatula* chitosanase activities by Davis (A) and Reisfeld (B) native electrophoresis. 20 µg of proteins from control (C), *G. mosseae*-inoculated roots (Gm) and nodules (N) were loaded in each lane. Chitosanase activities were revealed by Calcofluor and Coomassie blue double-staining, appearing as white bands against a blue background.

and Coomassie blue staining (Fig. 8). No chitosanase isoform was found in control roots. In *G. mosseae*-inoculated roots, 3 isoforms behaving with apparent molecular weights of 18, 19 and 21 kDa were detected. Like the mycorrhiza-related chitinases, these isoforms were induced since 14 days after inoculation, and their activity was enhanced along time (data not shown). None of these isoforms was induced neither by *A. euteiches* nor by the elicitor treatments (data not shown). They were not found either in *S. meliloti*-inoculated roots, but one chitosanase isoform with an apparent molecular weight of 19 kDa was found in isolated nodules (Fig. 8).

To get more information about the induction of these chitosanase isoforms, protein extracts from control roots, *G. mosseae*-inoculated roots and isolated nodules were separated on Davis and Reisfeld native gels (Fig. 9). In Davis gels (Fig. 9A), 3 bands were detected in mycorrhizal roots, but none in nodules. In Reisfeld gels (Fig. 9B), 2 major bands were found in mycorrhizal roots, and 2 isoforms were also found in nodules. Although these isoforms did not migrate to the same level, they could correspond to those observed in mycorrhizal roots: indeed, in this native system where the substrate is embedded in the gel matrix, proteins also migrate by affinity and the more enzyme there is, the further it migrates. Based on this assumption, these 2 isoforms could correspond to the 19 kDa isoform detected by SDS-PAGE in both *G. mosseae*- and *S. meliloti*-inoculated roots.

4. Discussion

The induction of specific mycorrhiza-related isoforms appears a general phenomenon, since they were reported to occur in several plants such as tobacco, pea, onion, tomato or soybean (Dumas-Gaudot et al., 1992a; 1992b; 1994; Pozo et al., 1996; Xie et al., 1999). In the present work, such isoforms were found to be induced in the model plant *Medicago truncatula* by the arbuscular mycorrhizal fungus *Glomus mosseae*, with isoelectric points ranging from 5.3 to 5.8 and apparent molecular weights of 29 to 40 kDa. Immunoblotting with several antibodies revealed the cross-reactivity of the 30 kDa isoforms with a basic class I chitinase isolated from winged bean. Again these results concord with those obtained on pea, in which one of the mycorrhiza-related isoforms has recently been purified and partially sequenced, showing strong homology to a basic class I chitinase expressed in pea shoots (Slezack et al., 2001). Class I chitinases are usually located in the vacuole, but vacuolar targeting appears to be mediated by a C-terminal extension that is missing in acidic chitinases (Chrispeels and Raikhel, 1992). Therefore it is not surprising that the 30 kDa mycorrhiza-related isoforms detected in our study, which could correspond to the acidic isoforms detected in Davis and isoelectricfocusing gels, are

immunologically related to a basic class I chitinase. Strikingly, these isoforms also cross-reacted with an antibody raised against a class III chitinase from chickpea. Class III chitinases are mostly localized in the extracellular compartment and are often found to display lysozyme activity (Graham and Sticklen, 1994). Originally refereeing to isoforms showing homology with bacterial and fungal chitinases, class III chitinases were first said to bear no homology with class I chitinases (Collinge et al., 1993). However, comparison of consensus sequences later revealed some similarity between class I and class III chitinases (Levorson and Chlan, 1997). The 40 kDa isoform only cross-reacted with the chickpea class III antibody. Interestingly, the expression of some class III mycorrhiza-specific chitinase genes was demonstrated to be induced in the late stages of *M. truncatula -G. mosseae* symbiosis (Salzer et al., 2000). However, it is not possible to conclude whether the product of these genes correspond to the 40 kDa isoform detected in our study, since the complete sequences, that would allow the calculation of some molecular weight data, are not available.

In our study, the mycorrhiza-related chitinase isoforms were detected since 14 days after inoculation and their activity increased along time, concording with the increase in colonization intensity and amount of arbuscules. This is in agreement with the hypothesis that these isoforms play a role in the formation and/or the senescence of arbuscules. Indeed, their induction did not occur neither in mycorrhizal pea treated with gibberellic acid, which inhibits the formation of these symbiotic structures, nor in some pea mutant deficient for mycorrhization (Slezack et al., 2000). Furthermore, the occurrence of N-acetylglucosamine residues (the monomeric form of chitin) has been reported on the arbuscule cell wall (Jabaji-Hare et al., 1990). Recently, further evidence of such role was brought with the specific localization of some chitinase transcripts in arbuscule-containing cells of *M. truncatula* (Bonanomi et al., 2001).

As a goal to better characterize the mycorrhiza-related isoforms induced in *M. truncatula* roots, plants were inoculated with several microorganisms such as the nitrogen fixing symbiont *S. meliloti* or the pathogenic fungus *A. euteiches*, as well as with another mycorrhizal fungus, *G. intraradices*. When extracts were separated in Davis gels, the mycorrhiza-related isoforms could only be detected in mycorrhizal roots, but some new chitinase isoforms were found in nodules and in pathogen-treated roots. Induction of chitinases upon pathogenic infection has been widely documented, and *in vitro* antifungal activity of plant chitinases has been shown with the degradation of fungal cell walls and the inhibition of fungal growth at hyphal tips (Mauch et al., 1988; Schlumbaum et al., 1986). In response to rhizobial symbiosis, elevated levels of plant chitinase activity have been suggested to account for the degradation of Nod factors

produced by the bacteria or to play a role in the morphological events taking place during the development of nodules (Staehelin et al., 1994).

In mutant plants inoculated with *G. mosseae*, the 3 mycorrhiza-related isoforms were detected, although very faintly. Since no colonization was achieved in this mutant, this result encounters the hypothesis of a unique role of the mycorrhiza-related isoforms in arbuscule turnover. It constrasts with the results obtained in pea where no induction could be observed in a mycorrhiza-deficient mutant (Slezack et al., 2000). However, on the contrary to pea, appressoria observed at the root surface of *M. truncatula* mutants were complex and ramified. This feature has already been described in *M. sativa* (Bradbury et al., 1991), tomato (Barker et al., 1998) and *Lotus japonicus* (Senoo et al., 2000) mutants, as well as in another mutant of *M. truncatula* (Calantzis et al., 2001). Since recently it was shown that the application of chitinase induce the alteration (wave-pattern and swelling) of *G. mosseae* hyphal tips (Vierheilig et al., 2001), we may assume that the mycorrhiza-related isoforms detected in our study are involved in the branching of fungal hyphae, either at the root surface for the formation of ramified appressoria in mutant plants, or inside the cortical root cells for the development of arbuscules.

One very acidic chitinase was detected early and transiently in *G. mosseae*- and *S. meliloti*-inoculated roots. The induction and subsequent suppression of defense related genes and products have already been reported in response to mycorrhizal and rhizobial symbioses (Cook et al., 1995; Harrison and Dixon, 1994; Kapulnik et al., 1996; Lambais and Medhy, 1993; Spanu et al., 1989; Vasse et al., 1993). In particular, the transcripts of one acidic class III chitinase were found to accumulate in *Sesbania rostrata* stem-borne nodules from 2 to 7 days after inoculation with the bacteria *Azorhizobium caulinodans* (Goormachtig et al., 1998). The acidic isoform observed in the present work could be induced by Nod factors during the colonization of the root by *S. meliloti*, and to similar elicitor molecules produced by *G. mosseae*. The existence of such elicitors has already been postulated to occur in the mycorrhizal symbiosis (Gollotte et al., 1997) but none have been isolated so far. Indeed, the transient nature of this induction recalls the hypersensitive reaction taking place in incompatible plant-pathogen interactions, and might result from a control mechanism exerted either by the plant or the fungus (Dumas-Gaudot et al., 2000).

As for chitinases, newly-induced chitosanase isoforms were reported in onion, leek, tomato and pea upon mycorrhizal inoculation (Dumas-Gaudot et al., 1992b; Pozo et al., 1998; Slezack et al., 2000). The present study brings further evidence that this induction is a generalized phenomenon, with the detection of 3 chitosanase isoforms with apparent molecular weights between 18 to 21 kDa in *M. truncatula* roots inoculated with the arbuscular mycorrhizal fungus *G. mosseae*. Interestingly, the 19 kDa isoform was also detected in nodules isolated from *S. meliloti*-inoculated roots. Further characterization allowed to

identify 3 acidic isoforms present only in mycorrhizal roots, and 2 basic isoforms which were found in both interactions. Chitosanases have been reported to hydrolyze fungal cell wall polysaccharides (Grenier and Asselin, 1990) and they also are developmentally regulated (El Ouakfaoui and Asselin, 1992). The detected chitosanase isoforms could therefore be involved in morphological events linked to the development of arbuscules and nodules. These isoforms could also participate to a signalling pathway based on the degradation of chitosan oligomers. The 2 acidic isoforms specifically induced in mycorrhization could be more precisely involved in the formation of arbuscules, since their induction was found to concord with that of the mycorrhiza-related chitinase isoforms, being detected 14 days after infection and increasing along time. Chitin and chitosan are indeed both cell wall components of arbuscular mycorrhizal fungi (Bonfante-Fasolo and Grippiolo, 1984; Wessels and Siestma, 1981) and a combined action of chitinases and chitosanases in the formation and/or degradation of arbuscules appears highly probable. Further molecular work including identification of the corresponding genes and immunolocalization studies should help to elucidate the role of mycorrhiza-related chitinase and chitosanase isoforms.

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