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Symbiosis-stimulated chitinase isoenzymes of soybean (*Glycine max* (L.) Merr.)

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Abstract

Isoforms of endochitinase in soybean were studied in relation to root symbiosis. Five selected cultivars differing in their nodulation potential were inoculated with two strains of *Bradyrhizobium japonicum*, the broad host-range *Rhizobium* sp. NGR234, and with the mycorrhizal fungus *Glomus mosseae*. Total chitinase activity in nodules was up to 7-fold higher than in uninoculated roots and in mycorrhizal roots. The chitinase activity in nodules varied depending on the strain–cultivar combination. On semi-native polyacrylamide gels, four acidic isoforms were identified. Two isoforms (CH 2 and CH 4) were constitutively present in all analysed tissues. The other two isoforms (CH 1 and CH 3) were strongly induced in nodules and were stimulated in mycorrhizal roots as compared to uninoculated roots. The induction of CH 1 varied in nodules depending on the soybean cultivar. This isoform was also stimulated in uninfected roots when they were treated with tri-iodobenzoic acid, rhizobial lipochitooligosaccharides (Nod factors) and chitotetraose. CH 3 was not affected by these stimuli indicating that this isoform could represent a marker for enzymes induced in later stages of the symbiotic interactions.

Key words: (*Brady*)*rhizobium*, chitinase isoenzymes, mycorrhiza, (restricted) nodulation, Nod factors.

Introduction

Roots of legumes are able to form nodules containing nitrogen-fixing symbiotic rhizobia (Mylona *et al.*, 1995) and to be colonized by arbuscular mycorrhiza (AM) fungi (Harrison, 1997). Often, nodule formation by a given bacterial strain is restricted to only a few host plant genera, and varieties of a given legume species may differ in nodule formation when inoculated with a given strain. Specifically in soybean, allelic genes have been described which restrict nodulation of certain genotypes by certain *Bradyrhizobium* strains (Vest, 1970; Qian *et al.*, 1996). Other, 'broad host-range' rhizobia exist: *Rhizobium* sp. NGR234 is such an example, and it is able to induce nodules on more than 110 genera of legumes as well as the non-legume *Parasponia* (SG Pueppke and WJ Broughton, unpublished results). During the infection process, plants secrete flavonoids and isoflavonoids which induce bacterial nodulation genes. Most of these genes are involved in the synthesis of Nod factors, a family of lipo-chitooligosaccharide signal molecules. Nod factors stimulate various responses in the host plants, including root hair deformation, induction of early nodulins, and formation of nodule primordia (Dénarié *et al.*, 1996; Long, 1996; Schultze and Kondorosi, 1996).

In contrast to rhizobia, AM fungi usually have a 'broad host-range': i.e. a given species can usually form mycorrhizal roots with many different angiosperm families. Several similarities have been reported between the rhizobial symbiosis of legumes and the symbiotic interaction of plants with AM fungi. For example, non-nodulating legume mutants are sometimes unable to be colonized by mycorrhizae (Duc *et al.*, 1989; Bradbury *et al.*, 1991), certain nodulins are induced by AM fungi (Wyss *et al.*, 1990; van Rhijn *et al.*, 1997) and it was also

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Abbreviations: AM, arbuscular mycorrhiza; chitotetraose, *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose; FW, fresh weight; NNP, normal nodulation potential; Nod, nodulation; RNP, restricted nodulation potential; TIBA, tri-iodobenzoic acid.

found that the rhizobial Nod factors stimulate the mycorrhizal colonization of soybeans suggesting common events in early recognition processes (Xie *et al*., 1995).

Chitinase isoforms are not only thought to play a role in the defence of plants against pathogens, but also appear to have a function in mutualistic symbioses. Roots infected by AM fungi often show a transient stimulation of chitinase activity and corresponding mycorrhiza-induced isoenzymes have been identified (Spanu *et al*., 1989; Dumas-Gaudot *et al*., 1992, 1996; Volpin *et al*., 1994). In the context of nodule symbiosis, chitinases have been described to inactivate the Nod factors by cleaving the chitooligosaccharide backbone (Staehelin *et al*., 1994*a*, *b*). Chitinase activity is stimulated in the nodule cortex and in the infected zone of some ineffective soybean nodules (Staehelin *et al*., 1992; Parniske *et al*., 1994). A chitinase has been localized in aborted infection threads of *Medicago* roots (Vasse *et al*., 1993) and a chitinase gene was induced during stem nodule development of *Sesbania* (Goormachtig *et al*., 1998). In soybean roots, two different ethylene-induced chitinase isoenzymes were found, and it was proposed that they may be involved in the inhibition of nodule development by ethylene (Xie *et al*., 1996).

In the present work, chitinase isoforms from five soybean cultivars infected by three rhizobial strains and by a mycorrhizal fungus were investigated. Two symbiosis-induced chitinases were identified in nodules and mycorrhizal roots. One of them was also stimulated in uninfected roots treated with TIBA, Nod factors or chitotetraose.

Materials and methods

Biological materials

Seeds of 92 soybean cultivars [*Glycine max* (L.) Merr.] were kindly provided by the Chinese Academy of Agricultural Science (Beijing, People's Republic of China). Based on a preliminary analysis, two cultivars with a normal nodulation potential, namely the cultivars 'An Tu Bai-Hua Lu Da Dou' and 'Gong Jiao-6308-1' (Xie *et al*., 1996), and two cultivars with a reduced nodulation potential, namely 'Dong Da Li' and 'Du Lu Huang', were selected for detailed studies (Table 1). *Glycine max* cv. 'Maple Arrow', a cultivar with normal nodulation potential, was also included and was provided by Semences UFA (Busigny, Switzerland). All seeds were surface-sterilized with 30% H_2O_2 for 20 min, followed by washing with sterile tap-water, and incubated at 27 °C on 1% water agar plates for germination. The bacterial strains *Bradyrhizobium japonicum* 61-A-101 (Staehelin *et al*., 1992), *Bradyrhizobium japonicum* USDA110*spc*4 (Regensburger and Hennecke, 1983), and *Rhizobium* sp. NGR234 (Rif^R) (Lewin *et al*., 1990), were grown to stationary phase in 20E-medium (Werner *et al*., 1975) at 27 °C on a rotary shaker at 140 rpm. Inoculum of the mycorrhizal fungus *Glomus mosseae* (Nicol. and Gerd.) was prepared from the host plant *Plantago lanceolata* as described previously (Vierheilig *et al*., 1993).

Establishment of the symbioses

One-week-old soybean seedlings individually grown in 'Leonard jars' filled with sterile perlite and vermiculite (1:1, v/v) were inoculated with 5 ml rhizobial cultures of the indicated strains as described (Staehelin *et al*., 1992). In mycorrhizal experiments, soybeans were individually grown in 150 ml pots containing the fungal inoculum, sand, and loam in the ratio 1:1:1 (by vol.); Xie *et al*., 1995). For both symbioses, uninoculated roots served as a control. Leonard jars and pots were cultivated in a phytotron (14 h day at a photon flux of 300 μmol m^{-2} s^{-1} and 26 °C, 10 h night at 20 °C). Roots and nodules were harvested after 3–4 weeks of co-cultivation and their FW was determined. Plant material was stored at −20 °C until extraction. Staining of roots with trypan blue and the estimation of mycorrhizal colonization was performed as described earlier (Xie *et al*., 1995).

Treatment of plants with chemical stimuli

Three-day-old soybeans, cv. 'Maple Arrow', germinated on water agar plates (*c*. 10 per plate), were individually transferred into sterilized 'Magenta jars'. The upper jar was filled with sterile perlite and vermiculite (1:1, w/w) (upper jar) and the lower jar with 200 ml B+D medium (Broughton and John, 1979). Chemical stimuli, either 50 μM tri-iodobenzoic acid (TIBA) (Fluka, Buchs, CH), 0.1 μM purified acetylated Nod factors (NodNGR-V(MeFuc,Ac) from *Rhizobium* sp. NGR234 (Price *et al*., 1992), or 0.1 μM *N*,*N*′,*N*″*N*‴tetraacetyl-chitotetraose (pure grade, Seikagaku Corporation, Tokyo, Japan) were added to the nutrient solution where appropriate. Roots were harvested after 4 weeks, immediately frozen, and stored at −20 °C until further analysis.

Preparation of enzyme extracts

Frozen plant material was ground in ice-cold morpholino-ethanesulphonic acid (potassium salt) buffer (0.1 M, pH 6.3) containing ethylenediaminetetraacetic acid and phenylmethyl-sulphonylfluoride (2 mM each), and centrifuged at 20 000 *g* for 10 min. Aliquots of the supernatant, corresponding to the FW of the plant material, were used for the endochitinase assay and the separation of chitinase isoforms.

Endochitinase assay with [^{3}H]-chitin

Total endochitinase activity was assayed at pH 5 using colloidal [^{3}H]-chitin as substrate (Boller *et al*., 1983). After stopping the reaction with trichloroacetic acid, the released soluble chitooligosaccharides were radiometrically determined. One nkat was defined as the activity that generates soluble chito-oligosaccharides corresponding to 1 nmol *N*-acetyl-glucosamine in 1 s.

Separation and detection of chitinase isoforms

Chitinase isoforms were separated at 4 °C under semi-native conditions according to the method of Trudel and Asselin (1989) using 10% (w/v) acrylamide gels containing 0.1% SDS and 0.01% (w/v) glycol chitin. After incubation in acetate (sodium salt) buffer (pH 5, 100 mM) containing 1% (v/v) Triton-X-100 for 1 h at 37 °C, gels were stained with 0.01% fluorescent brightener (Calcofluor White, Sigma) and analysed under UV-radiation at 354 nm. The chitinase isoforms were seen as dark bands against a brightly fluorescing background.

Results

Characterization of the symbiotic performance of different soybean cultivars

In a preliminary experiment, a total of 92 soybean cultivars commonly used in the People's Republic of China were tested for their symbiotic capacity with *Bradyrhizobium japonicum* 61-A-101, a strain known to nodulate soybean cv. 'Maple Arrow' efficiently (Staehelin *et al.*, 1992). Although most cultivars formed many nodules, 15 varieties were characterized by a restricted nodulation (five or fewer nodules per root system) and defined as 'RNP cultivars'. For this study, two of these RNP cultivars were used, namely 'Dong Da Li' and 'Du Lu Huang' and three cultivars with a normal nodulation potential (NNP) which included the Canadian cultivar 'Maple Arrow', 'An Tu Bai-Hua Lu Da Dou', and cv. 'Gong Jiao-6308-1' which responds strongly to ethylene (Xie *et al.*, 1996).

The symbiotic capacity of these five cultivars was tested by inoculating them with *B. japonicum* 61-A-101, *B. japonicum* USDA110 and by the 'broad host-range' strain *Rhizobium* sp. NGR234, and furthermore with the mycorrhizal fungus *Glomus mosseae* (Table 1). The NNP cultivars were well nodulated by both bradyrhizobial strains. In contrast, the two RNP cultivars 'Dong Da Li' and 'Du Lu Huang' which had only a restricted nodulation potential with respect to strain 61-A-101 had an almost normal nodulation potential with strain USDA110. *Rhizobium* sp. NGR234 induced bacteria-free root out-growths called 'pseudonodules' on all five cultivars and occasionally ineffective (fix^-) nodules, as determined by the acetylene reduction method (data not shown). *Glomus mosseae* colonized the roots of all cultivars to a similar degree, indicating that mycorrhization is not hindered in the RNP cultivars 'Dong Da Li' and 'Du Lu Huang' (Table 1).

Table 1. *Characterization of the symbiotic capacity of three NNP and two RNP soybean cultivars*

Plants were inoculated either with the indicated (*Brady*)*rhizobium* strains or in a separate experiment with the AM fungus *Glomus mosseae*. Plants were harvested 4 weeks after inoculation. Their symbiotic capacity was characterized by determining the number of effective nodules (strains *B. japonicum* 61-A-101 and USDA110), the number of ineffective (pseudo)nodules (strain *Rhizobium* sp. NGR234) and by determining the degree of mycorrhizal root colonization. The mean values ± SE are given for three independent plants.

Cultivar	Nodulation		Mycorrhizal colonization (% of root length)
	Strain	Nodules[a] per plant	
NNP cultivars			
An Tu Bai-Hua Lu Da Dou	61-A-101	152 ± 9	50.0 ± 2.4
	USDA110	64 ± 11	
	NGR234	≫200	
Gong Jiao-6308-1	61-A-101	46 ± 10	51.0 ± 6.2
	USDA110	77 ± 8	
	NGR234	38 ± 7	
Maple Arrow	61-A-101	92 ± 22	35.0 ± 5.7
	USDA110	67 ± 10	
	NGR234	8 ± 2	
RNP cultivars			
Dong Da Li	61-A-101	< 5	44.5 ± 6.5
	USDA110	60 ± 23	
	NGR234	4 ± 1	
Du Lu Huang	61-A-101	< 5	43.7 ± 4.5
	USDA110	52 ± 15	
	NGR234	3 ± 3	

[a]A mixture of ineffective nodules and nodule-like structures ('pseudonodules') in the case of *Rhizobium* sp. NGR234.

Induction of chitinase activity in nodules

As reported previously for cv. Maple Arrow, chitinase activity was found to be enhanced in nodules compared to uninoculated roots (Staehelin *et al.*, 1992). Here chitinase activity was examined in roots and nodules of five different soybean genotypes (Table 2). The ability to release chitooligosaccharides from chitin was increased in all tested functional nodules induced by *B. japonicum*, and to a similar degree in the non-functional nodules induced by *Rhizobium* sp. NGR234, as compared to uninfected roots. For most cultivars, the increase of chitinase activity in nodules was dependent on the strain–cultivar combination analysed. Highest chitinase activity was measured for the 'Dong Da Li' nodules containing strain USDA110 which showed a 7-fold increase compared to uninfected roots. Values of chitinase activity in roots or nodules from the RNP cultivars 'Dong Da Li' and 'Du Lu Huang' were of the same order of magnitude as those of the three NNP cultivars. A correlation between chitinase activity and nodule number could not be detected. Mycorrhizal roots exhibited chitinase activities which were not significantly increased compared to uninfected roots (data not shown).

Symbiosis-related chitinase isoenzymes

As the increase of chitinase activity in soybean nodules is possibly due to the induction of specific isoforms, equal quantities of plant extracts (corresponding to the same FW) were analysed by electrophoresis on semi-native polyacrylamide gels using glycol chitin as the substrate. On typical chitinase isoenzyme profiles from roots and nodules of cv. 'Maple Arrow', four isoforms were identified as distinct bands and named CH1, CH2, CH3, and CH4 (Fig. 1). Occasionally, a weak band (CH1′) was seen. However, the detection of CH1′ in a given extract was not observed in all electrophoretic experiments (not shown) suggesting an 'unstable' enzyme activity under the conditions tested.

In all samples of soybean cultivars examined, CH2 and CH4 were constitutively present with similar activities in uninfected roots, mycorrhizal roots, nodules and pseudonodules (Fig. 1, lanes 1–4). In contrast to these

Table 2. *Chitinase activity in uninoculated roots, effective nodules (strains* B. japonicum *61-A-101 and USDA110) and ineffective (pseudo) nodules (strain* Rhizobium *sp. NGR234)*

The soybeans were either axenically cultivated or inoculated by the indicated (*Brady*) *rhizobium* strains. Roots or nodules were harvested 3–4 weeks later. Chitinase activity was measured by determining the amount of released oligosaccharides from colloidal ^{3}H-chitin. The mean values ± SE are given for three plants. (n.d. = not determined).

Cultivar	Chitinase activity (nkat g^{-1} FW)			
	Roots	Nodules[a] induced by strain		
		61-A-101	USDA110	NGR234
NNP				
An Tu Bai- Hua Lu Da Dou	1.1 ± 0.4	2.8 ± 0.9	3.9 ± 0.1	1.9 ± 0.5
Gong Jiao-6308–1	1.8 ± 0.2	5.5 ± 0.9	5.1 ± 1.3	5.1 ± 2.3
Maple Arrow	0.6 ± 0.3	2.8 ± 0.5	1.4 ± 0.1	3.2 ± 0.6
RNP				
Dong Da Li	1.7 ± 0.5	4.9 ± 0.3	11.1 ± 0.3	5.0 ± 1.1
Du Lu Huang	0.5 ± 0.1	1.2 ± 0.4	2.7 ± 0.3	1.8 ± 1.5

[a]A mixture of ineffective nodules and nodule-like structures in the case of *Rhizobium* sp. NGR234.

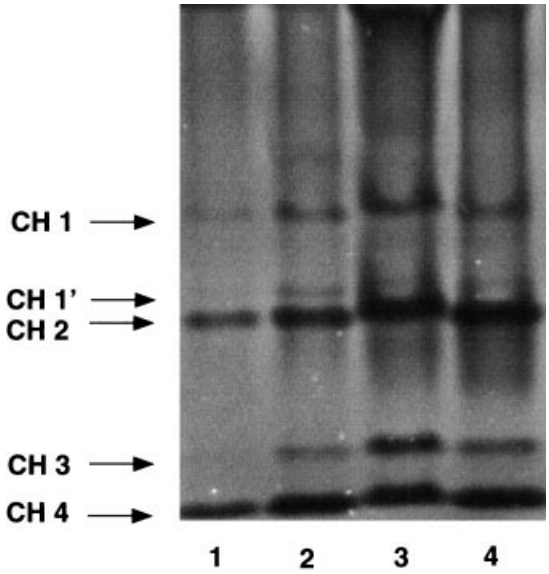

Fig. 1. Characterization of chitinase isoforms (CH) of *Glycine max* cv. 'Maple Arrow' after gel electrophoresis on polyacrylamide gels containing glycol chitin. Gels were incubated at 37 °C for 1 h and than stained with Calcofluor White. Lytic zones were visualized by UV illumination. Samples corresponding to 5 µg (FW) were obtained from the following material: sterile roots (lane 1), roots colonized by *Glomus mosseae* (lane 2), nodules induced by *B. japonicum* 61-A-101 (lane 3) and (pseudo) nodules induced by *Rhizobium* sp. NGR234 (lane 4).

constitutive isoforms, the intensity of the CH1 and CH3 bands varied in the samples of cv. 'Maple Arrow' (Fig. 1) analysed. In uninfected roots, CH1 and CH3 were only visible as very faint bands (Fig. 1, lane 1). However, these two isoforms were strongly induced in nodules containing strain *B. japonicum* 61-A-101 (Fig. 1, lane 3) and, to a lesser extent, in (pseudo)nodules induced by *Rhizobium* sp. NGR234 as well (Fig. 1, lane 4). In mycorrhizal roots, CH1 and CH3 were found to be stimulated as well, but to a lesser extent than in nodules (Fig. 1, lane 2). These data suggest the presence of two symbiosis-related soybean chitinases induced in both rhizobial and mycorrhizal symbioses. Moreover, strong non-migrating activity was observed at the top of the gel, indicating the presence of at least one additional symbiosis-stimulated chitinase isoform.

To check whether the induction of CH1 and CH3 occurs in a cultivar-specific manner, roots and nodules from various cultivar–strain combinations were analysed. Uninfected roots from all five cultivars showed similar isoenzyme profiles characterized by the two constitutive isoforms CH2 and CH4 (Fig. 2). However, genotype-specific differences were found with regard to the symbiosis-induced chitinase isoform CH1, while the intensity of the CH3 band was similar for all nodules tested

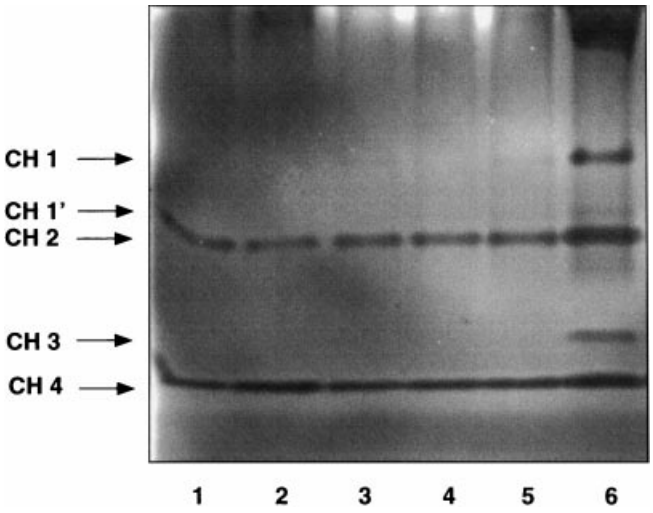

Fig. 2. Chitinase isoenzymes in sterile roots of *Glycine max* NNP cv. 'An Tu Bai-Hua Lu Da Dou' (lane 1), RNP cv. 'Dong Da Li' (lane 2), RNP cv. 'Du Lu Huang' (lane 3), NNP cv. 'Gong Jiao-6308-1' (lane 4), and NNP cv. 'Maple Arrow' (lane 5). A nodule extract obtained from 61-A-101-infected 'Maple Arrow' plants was used for comparison (lane 6). Chitinase isoforms were analysed as described in Fig. 1.

containing *B. japonicum* 61-A-101 or USDA110. In nodules of the NNP-cultivar 'An Tu Bai-Hua Lu Da Dou' for example, CH1 was seen as a weak band (Fig. 3, lanes 1, 2). Nodules of the RNP-cultivar 'Dong Da Li' were characterized by high CH1 activity, independently of the symbiotic bradyrhizobial strain (Fig. 3, lanes 3, 4). CH1 was also strongly induced in the RNP cultivar 'Du Lu Huang', while CH1 in nodules of NNP cultivar 'Gong Jiao-6308-1' was poorly stimulated (data not shown).

Stimulation of CH1 in roots by chemical stimuli

Are chitinase isoforms induced by chemical compounds known to trigger symbiotic responses on roots? To answer this question, an auxin transport inhibitor (Hirsch *et al.*, 1989; Xie *et al.*, 1998; Mathesius *et al.*, 1998) and Nod factors, the lipo-chitooligosaccharide signals of (*Brady*)*rhizobium*, were added to the nutrient solution at concentrations known to be active on roots. As shown in Fig. 4, untreated control roots of cv. 'Maple Arrow' were characterized by the two constitutive activities, CH2 and CH4 (Fig. 4, lane C). However, when the plants were cultivated in solutions containing the auxin transport inhibitor tri-iodobenzoic acid or the lipo-chitooligosaccharide NodNGR-V(MeFuc,Ac) from *Rhizobium* sp. NGR234, a stimulation of CH1 (together with the 'unstable' band CH1') was observed, while CH3 was not induced by TIBA or Nod factors (Fig. 4, lane T, NF). The effect of chitotetraose, an elicitor which can be released from chitin-containing fungal cell walls, was also tested. Roots cultivated in a solution containing chitotetraose had a similar isoenzyme profile as those treated with TIBA or NodNGR-V(MeFuc,Ac) showing a stimulation of CH1 (and of CH1') compared to untreated control roots (Fig. 4, lane CT).

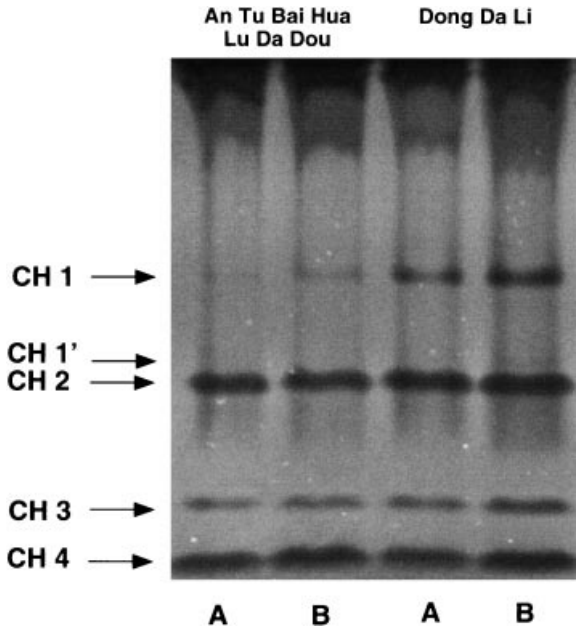

Fig. 3. Chitinase isoenzymes in nodules of *Glycine max* NNP cv. 'An Tu Bai-Hua Lu Da Dou' and of RNP cv. 'Dong Da Li'. Plants were either inoculated by *B. japonicum* 61-A-101 (A) or by *B. japonicum* USDA110 (B). Chitinase isoforms were analysed as described in Fig. 1.

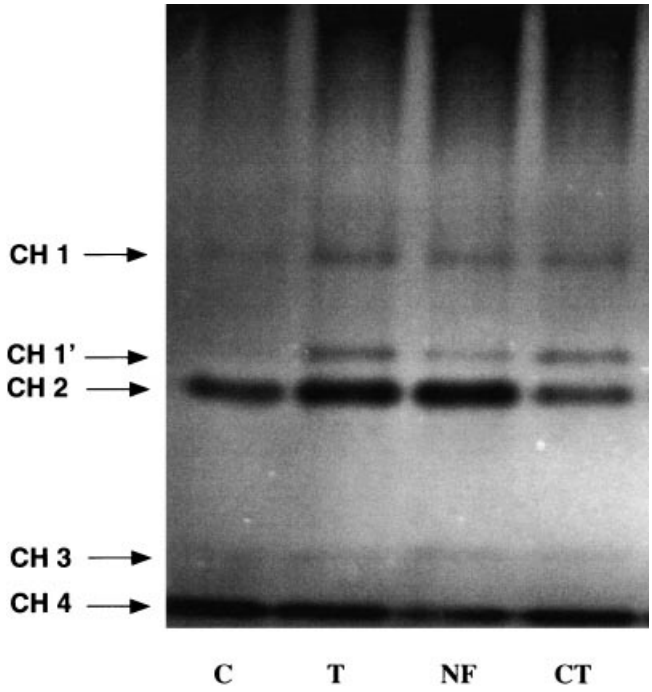

Fig. 4. Stimulation of CH1 (and CH1′) in uninoculated roots of soybean cv. 'Maple Arrow' by chemical stimuli. Plants were cultivated in a nutrient solution containing either 50 μM TIBA (lane T), 0.1 μM NodNGR-V(MeFuc,Ac), a Nod factor purified from *Rhizobium* sp. NGR234 (lane NF) or 0.1 μM chitotetraose (lane CT). Non-treated roots served as a control (C). Chitinase isoforms were analysed as described in Fig. 1.

Discussion

In the present paper, symbiosis-related chitinase isoenzymes of three cultivars with a normal nodulation potential and of two soybean cultivars which restrict nodulation by *Bradyrhizobium japonicum* 61-A-101 were investigated. High levels of total chitinase activity were found in nodules. Moreover, two acidic chitinases, CH1 and CH3, were specifically induced. These two isoenzymes were also found to be stimulated in roots colonized by mycorrhizal fungi. This indicates a host plant response that is common in both symbioses. In pea plants, however, chitinase isoenzymes have been reported to be differently stimulated in nodules and AM roots (Dumas-Gaudot *et al.*, 1996).

Interestingly, the two symbiosis-related chitinase isoforms of soybean differed with respect to their inducibility. CH1 levels varied depending on the soybean genotype and were particularly high in nodules of the two RNP cultivars. However, symbiosis-related induction of CH1 was also found in cultivars having a normal nodulation potential. Therefore, it is premature to conclude that CH1

is controlled in parallel to the restriction of nodule development in RNP cultivars.

In uninoculated roots, CH1 was elicited by the auxin-transport inhibitor TIBA, an observation which was made recently for chitinases in *Lablab purpureus* roots (data not shown). Moreover, CH1 was stimulated by rhizobial lipochitooligosaccharides (Nod factors), and by chitotetraose. This stimulatory effect suggests a role for CH1 in early and perhaps transient feedback processes. In the rhizobial symbiosis, CH1 could be involved in degradation and inactivation of Nod factors after their perception, as shown for a Nod factor-degrading hydrolase from *Medicago sativa* (Staehelin *et al*., 1995). In the AM symbiosis, CH1 could be involved in degradation of chitin fragments released through arbuscular turnover, thus preventing the induction of plant defence reactions. Chitin oligomers have been shown to elicit defence responses in various plants(see for review Boller, 1995), especially when suspension-cultured cells were treated with these compounds (Ren and West, 1992; Felix *et al*., 1993; Savouré *et al*., 1997; Kaku *et al*., 1997).

CH3, the other symbiosis-related acidic chitinase, was not stimulated in roots by the tested compounds and its level in nodules was similar in all five genotypes tested. This isoenzyme can be considered as a marker enzyme for the established stage of symbiosis. It remains to be analysed whether CH3 is also induced by pathogens or whether its stimulation is restricted to a developmental programme activated exclusively by mutualistic micro-organisms. Furthermore, it is worthwhile investigating whether CH1′ is really differentially regulated or whether it is the degradation product of another chitinase isoform. It will also be of interest to know what corresponds to the non-mobile chitinase activites on top of the gels. For these activities, another gel system has to be developed. Taken together, specific soybean chitinases have been identified that are induced in nodules and in roots colonized by AM fungi. The differential inducibility of these enzymes are in line with the hypothesis that specific chitinases are involved in complex feedback mechanisms at different symbiotic stages.

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