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Chitinase and peroxidase in effective (fix^+) and ineffective (fix^-) soybean nodules

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Abstract. Chitinase and peroxidase, two enzymes thought to be involved in the defense of plants against pathogens, were measured in soybean (*Glycine max* L. Merr.) roots and in nodules colonized by *Bradyrhizobium japonicum* strains differing in their symbiotic potential. Activities of both enzymes were higher in nodules than in roots. In "effective", nitrogen-fixing nodules, colonized by wild-type bacteria, chitinase and peroxidase activities had low levels in the central infected zone and were enhanced primarily in the nodule cortex. An ascorbate-specific peroxidase, possibly involved in radical scavenging, had similarly high activities in the infected zone and in the cortex. "Ineffective" nodules colonized by bacteria unable to fix nitrogen symbiotically showed a similar distribution of chitinase and peroxidase. In another type of "ineffective" nodule, colonized by a *B. japonicum* strain eliciting a hypersensitive response, activities of both enzymes were enhanced to a similar degree in the infected zone as well as in the cortex. Tissue prints using a direct assay for peroxidase and an antiserum against bean chitinase corroborated these results. The antiserum against bean chitinase cross-reacted with a nodule protein of M_r 32 000; it inhibited most of the chitinase activity in the nodules but barely affected the chitinase in uninfected roots. It is concluded that proteins characteristic of the defense reaction accumulate in the cortex of nodules independently of their ability to fix nitrogen, and in the entire body of hypersensitively reacting nodules.

Key words: *Bradyrhizobium* – Chitinase – *Glycine* (nodules) – Hypersensitive reaction – Peroxidase (isoenzymes)

Introduction

The interaction of legumes with rhizobia normally culminates in the formation of a symbiotic organ, the nodule, which functions in nitrogen fixation (Rolfe and Gresshoff 1988; Sprent 1989). The early stages of infection by the rhizobia resemble a pathogen attack; the process of nodulation has therefore been viewed as a "beneficial disease", (Vance 1983) caused by a "refined pathogen" (Djordjevic et al. 1987).

It is interesting to study the extent to which inducible defenses against pathogens come into play during nodule formation. A classical defense reaction, the accumulation of phytoalexins, has been examined in soybean roots infected by *Bradyrhizobium japonicum* (Werner et al. 1985). Wild-type bacteria, which caused the formation of effective, nitrogen-fixing nodules, induced the formation of only small amounts of the soybean phytoalexin glyceollin at the earliest stages of interaction. However, certain strains which lead to the formation of ineffective nodules not fixing nitrogen, induced a sort of hypersensitive reaction and caused accumulation of large amounts of glyceollin in a way similar to roots infected by pathogens (Werner et al. 1985; Parniske et al. 1990).

We have thus studied further defense reactions in effective and ineffective nodules, focussing our attention on the activities of two enzymes often associated with plant defense reactions, namely chitinase (Boller 1988) and peroxidase (Hammerschmidt et al. 1982; Svalheim and Robertsen 1990). Plant chitinases inhibit the growth of certain fungi (Schlumbaum et al. 1986) and often have lysozyme activity as well, indicating that they may function in defense against bacteria (see Boller 1988). Peroxidases have been suggested to be involved in the activation of preformed toxins and in lignification, two typical elements of the plant defense reaction (Lewis and Yamamoto 1990). We report that chitinase and peroxidase are present at elevated levels in the cortex but not in the central region of effective root nodules and in the whole body of certain ineffective nodules. These findings indicate that the constitutive expression of defense-related genes in cortical cells of effective root nodules may function in protection of the symbiotic organ from pathogen attack.

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Abbreviations: PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulphate

Materials and methods

Growth of plants and establishment of symbiosis. Seeds of soybean (*Glycine max* L. Merr.) cv. Maple Arrow (Semences UFA, Bussigny, Switzerland) were surface-sterilized, grown on agar plates and infected 4 d after germination with one of the following strains of *Bradyrhizobium japonicum*, grown to stationary phase in 20E-medium (Werner et al. 1975) at 27° C on a rotary shaker at 140 rpm: 61–A–101 (fix^+), yielding effective, nitrogen-fixing nodules (Stripf and Werner 1978), RH–31-Marburg (fix^-), producing ineffective nodules of normal appearance (Werner et al. 1984), and 61–A–24 (fix^-), causing the appearance of small, greenish ineffective nodules (Werner et al. 1980). Uninfected seedlings were obtained by treatment with uninoculated 20E-medium. Uninfected and infected seedlings were grown in sterilized Leonard jars (Leonard 1943) filled with perlite and nutrient solution (Werner et al. 1975) containing 1 mM KNO_3 in a phytotron (14-h day at a photon flux of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 26° C, 10-h night at 20° C).

Enzyme extraction. Where needed, the cortex and the central infected zone of harvested nodules were separated after three cycles of freezing and thawing by cutting the nodules in two halves with a razor blade and dissecting the tissue with two needles. Tissue from uninfected roots and nodule material was extracted with mortar and pestle in cold 0.1 M phosphate (Na^+) buffer, pH 7 (2 ml per g FW) for the chitinase assay, or in 50 mM phosphate (K^+) buffer, pH 7 (4 ml per g FW) containing polyvinylpolypyrrolidone (0.25 g per g FW) for the peroxidase assay, respectively. The extracts were centrifuged at 15 000 · *g* for 15 min, and the supernatants were used for enzyme measurement and gel electrophoresis.

Enzyme assays, protein. Chitinase was assayed radiometrically at pH 5 using [^{3}H]chitin as substrate (Boller et al. 1983). Where required, various amounts of antiserum against bean chitinase were added until maximum inhibition was reached; preimmune serum served as a control. Peroxidase was measured photometrically, either as ascorbate peroxidase by the rate of decrease in A_{265} in the presence of 0.25 M ascorbate (Asada 1984) or as guaiacol peroxidase by the rate of increase in A_{470} with 10 mM guaiacol (Dalton et al. 1987), using the constant linear increase after 2 min incubation in the reaction mixture to reduce the impact of the pseudoperoxidase activity of leghemoglobin (Puppo et al. 1980). Protein was determined by the method of Bradford (1976).

Immune blots. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels according to Laemmli (1970), transferred electrophoretically onto nitrocellulose membranes (Towbin et al. 1979) and labelled sequentially by addition of a monospecific antiserum (1:1500 dilution) against bean chitinase (Vögeli et al. 1988), followed by a goat anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase (Biorad, Richmond, Cal., USA). The blots were developed with 4-chloro-1-naphthol and H_2O_2 as specified by the supplier (Biorad).

Activity stains. Chitinase activity was detected after SDS–PAGE in a 15% polyacrylamide gel using the method of Trudel and Asselin (1989).

Peroxidase was analyzed on native gels, using 7.5% acrylamide in 0.375 M Tris (Cl^-), pH 9 for the gels and 0.192 M glycine, adjusted to pH 9.0 with Tris, as running buffer. The samples (equivalent to 2.5 mg FW) contained 10% (v/v) glycol and 10 $mg \cdot L^{-1}$ bromophenol blue. Electrophoresis was achieved at 10 mA and 4° C. Peroxidase activity was detected by staining the gels with 4-chloro-1-naphthol in Tris-buffered saline (TBS) (10 mM Tris (Cl^-) buffer (pH 7.4), 0.15 M NaCl) starting the reaction by addition of 1 mM H_2O_2.

Tissue prints. Nodules were cut with a razor-blade into two halves and directly blotted on a nitrocellulose membrane (Cassab and Varner 1987; Hendriks and van Loon 1990). The blots were stained with Amido Black for detection of proteins (Hendriks and van Loon 1990) and either with 4-chloro-1-naphthol and H_2O_2 in TBS following the specifications of Biorad or with 3-amino-9-ethylcarbazole and H_2O_2 at pH 7.3 (Graham et al. 1965) for detection of peroxidase activity, stopping the reaction with distilled water. For immunodetection the tissue prints were incubated for 1 h in SDS–PAGE sample buffer according to Laemmli (1970) without Bromophenol Blue to inactivate endogenous phosphatase, blocked with 5% (w/w) nonfat dry milk in TBS, and incubated overnight with antiserum against chitinase or preimmune serum (1:6000 dilution) in the refreshed solution. After washing with distilled water for 1 min and several times with TBS-Tween (0.5% v/v Tween 20 in TBS) the membranes were incubated for 1 h with a goat anti-rabbit IgG antibody coupled to alkaline phosphatase (Sigma, Deissenhofen, FRG; using the dilution recommended by the supplier) in TBS with 2.5% (w/w) nonfat dry milk. The membranes were washed with TBS-Tween and stained with 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Sigma) and Nitro Blue Tetrazolium according to Harlow and Lane (1988).

Results

The enzymatic activities of chitinase and peroxidase in nodules induced by an effective strain of *B. japonicum*, 61–A–101, depended on the age of the nodule. In young nodules an increase of activity per gram fresh weight and per gram protein was found, whereas in older nodules only the activities per nodule increased, indicating that the activities increase in parallel to the increase in nodule size (Fig. 1). The results on ascorbate peroxidase during nodule development were in agreement with those of Dalton et al. (1986) who found a good correlation with the increase of nitrogenase.

When uninfected roots were compared with nodules induced by different strains of *B. japonicum*, chitinase and ascorbate peroxidase were always found to be higher in nodules than in roots. Guaiacol peroxidase exhibited a different pattern: It had a comparatively low activity in nodules except in the hypersensitively reacting nodules induced by strain 61–A–24 (Fig. 2).

The tissue distribution of chitinase and peroxidase was studied in nodules induced by different strains of bacteria. The activities of chitinase and guaiacol peroxidase, calculated per gram fresh weight, were more than three times higher in the cortex than in the central infected zone of effective and ineffective nodules induced with the strains 61–A–101 or RH–31, respectively. In contrast, high activities of these enzymes were also found in the infected zone of the ineffective nodules induced with strain 61–A–24. Ascorbate peroxidase had similar activities in the cortex and in the infected zone of all nodules studied; part of the activity appeared to be lost during tissue separation (Table 1).

For ascorbate peroxidase, the specific activity with ascorbate as a substrate has been found to be approximately equal to the specific activity with guaiacol (Dalton et al. 1987). Thus, the infected zone of nodules induced by 61–A–101 and RH–31 contained primarily ascorbate peroxidase, as indicated by the similarity of the specific activities of peroxidase measured with ascorbate and guaiacol (Table 1). In the cortex of these nodules, however, and in the whole body of the hypersensitively reacting nodules induced by 61–A–24, peroxidase

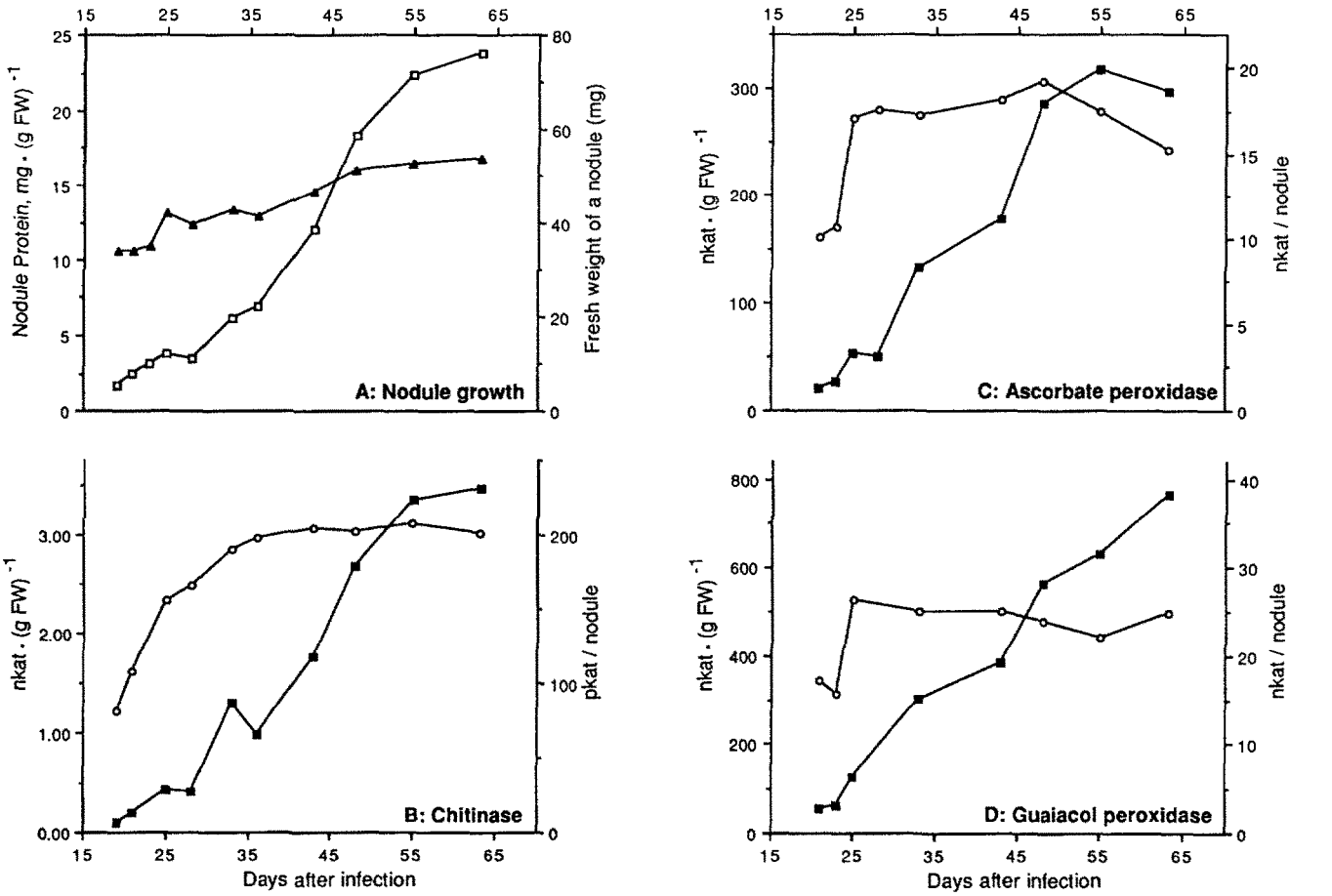

Fig. 1A–D. Growth of effective nodules and their activities of chitinase and peroxidase. Nodules were induced in soybean seedlings (4 d old) with *B. japonicum* strain 61–A–101, at day 0 and harvested at different times after infection. **A** Mean fresh weight (FW) of a single nodule (□) and its protein content on a fresh-weight basis (▲). **B, C, D** Enzyme activities, expressed per g FW (○) and per individual nodule (■). Chitinase activity (**B**) was determined radiometrically; peroxidase activity was measured photometrically with ascorbate (**C**) and guaiacol (**D**) as substrates

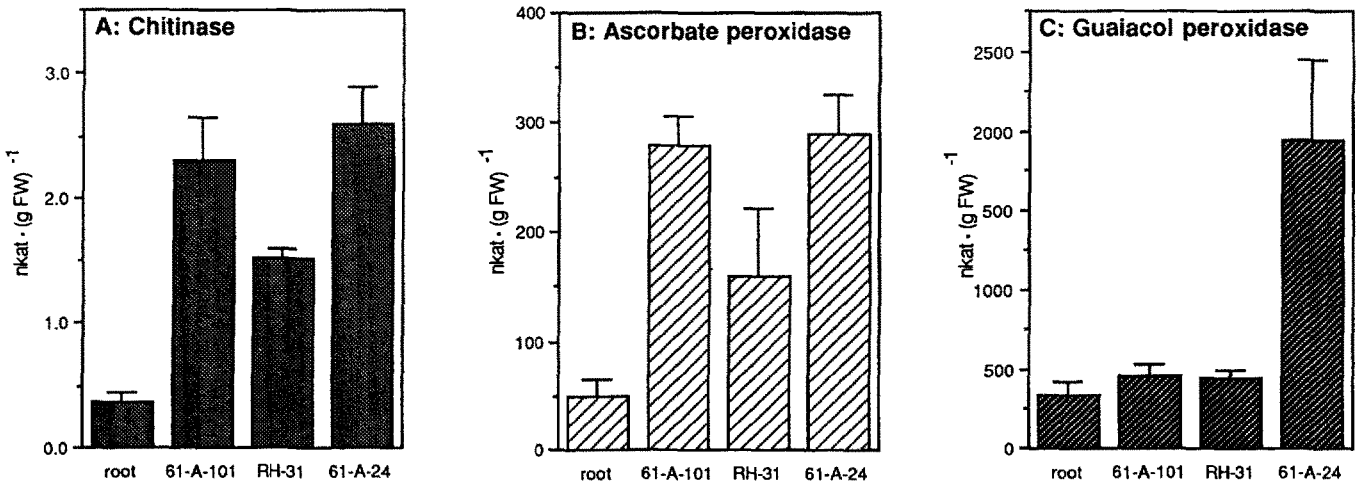

Fig. 2A–C. Chitinase (**A**) and peroxidase (**B, C**) activities in uninfected roots and in effective and ineffective nodules. Soybean seedlings (4 d old) were treated with uninoculated bacterial growth medium or with *B. japonicum*, strains 61–A–101, RH–31 or 61–A–24. Four weeks later, uninfected roots from mock-treated plants were compared with nodules from the infected plants

isoenzymes with a preference for guaiacol over ascorbate were present.

The existence of two different peroxidase isoenzymes was also apparent from electrophoretic analyses (Fig. 3). The band migrating more slowly was found to be localized mainly in the nodule cortex of effective nodules and in nodules induced by strain 61–A–24. On the basis of its localization, this is most probably the isoenzyme with a

Table 1. Enzyme activities of chitinase and peroxidase in the central infected zone and in the cortex of nodules 28 d after infection with *B. japonicum*, strains 61-A-101, RH-31and 61-A-24

Strain	Tissue	Enzyme activity (nkat · (g FW)$^{-1}$)[a]		
		Chitinase	Peroxidase	
			Ascorbate	Guaiacol
61-A-101	Infected zone	0.99 ± 0.21	289 ± 11	289 ± 16
	Cortex	3.72 ± 0.16	254 ± 46	940 ± 235
RH-31	Infected zone	0.79 ± 0.08	134 ± 35	164 ± 39
	Cortex	3.21 ± 0.18	126 ± 28	876 ± 243
61-A-24	Infected zone	2.48 ± 0.77	235 ± 13	2611 ± 420
	Cortex	3.64 ± 0.13	113 ± 36	1430 ± 236

[a] Mean and SD of three independent extracts

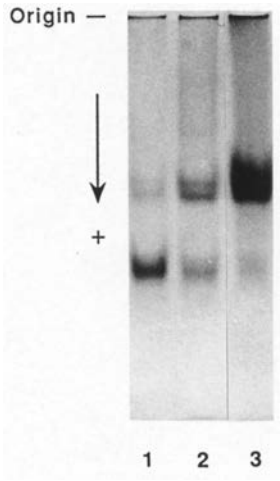

Fig. 3. Characterization of peroxidase isoenzymes after native gel electrophoresis. The following samples (corresponding to 2.5 mg FW) were subjected to electrophoresis: Extracts from infected zone *(lane 1)* and cortex *(lane 2)* of 28 d old soybean nodules induced with *B. japonicum*, strain 61–A–101; total extracts *(lane 3)* from nodules induced with *B. japonicum*, strain 61–A–24. Gels were stained with 4-chloro-1-naphthol in the presence of 1 mM H_2O_2

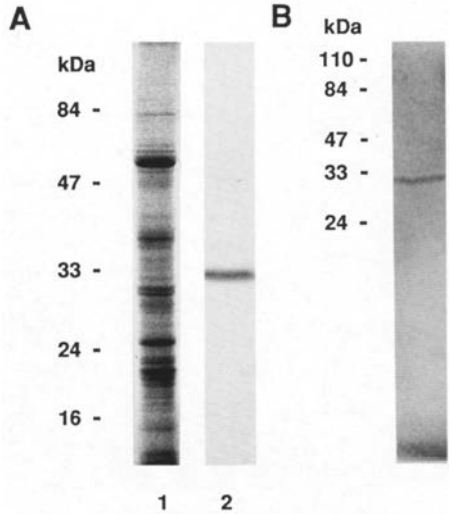

Fig. 4A, B. Characterization of chitinase after SDS–PAGE electrophoresis. **A** An extract from 28-d-old nodules induced with *B. japonicum*, strain 61–A–101 (40 µg protein) was subjected to SDS–PAGE. The proteins were blotted onto nitrocellulose and stained for proteins *(lane 1)* or immunodecorated with a rabbit antiserum against bean chitinase *(lane 2)*. **B** The same extract was subjected to SDS–PAGE in a gel containing 0.01% (w/v) glycol chitin and stained for chitinase activity. The position of molecular-weight markers is indicated on the *left*

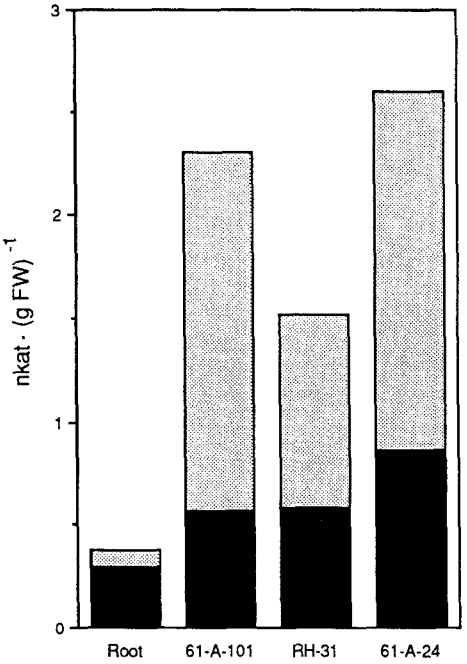

Fig. 5. Inhibition of root and nodule chitinase activities by an antiserum against bean chitinase. Uninfected roots and nodules induced with *B. japonicum*, strains 61–A–101, RH–31 and 61–A–24, were obtained as in Fig. 2. Extracts were incubated with or without antiserum against bean chitinase. Fractions of the total activity inhibited (▨) or not inhibited (■) by the antiserum are indicated. Mean standard deviations of the activities in the presence of antiserum were 4% of the total activities

preference for guaiacol. The more rapidly migrating band probably represents ascorbate peroxidase, the predominant peroxidase of the infected zone.

The serological properties of chitinase were studied using an antiserum against bean chitinase. Cross-reaction was found with various crude protein preparations from soybean. On immune blots prepared after SDS–PAGE, a protein band with an apparent M_r of 32000 reacted strongly with the antiserum (Fig. 4A). At the same position, chitinase activity could be detected in the SDS–PAGE gels using glycol chitin as substrate (Fig. 4B).

Extracts from 28-d-old nodules and from roots with comparable chitinase activities were incubated with various amounts of antiserum. The total chitinase activity decreased rapidly with the addition of small amounts of antiserum and reached a plateau. In nodule extracts, a large part of the activity was inhibited by the antiserum (Fig. 5). In contrast, chitinase activity in the roots was reduced only by a small fraction with the antiserum (Fig. 5). Preimmune serum did not affect chitinase activity. Using a soybean seed extract, inhibition of chitinase activity by the antiserum was nearly complete (data not shown).

The localization of the enzymes was further studied by tissue prints on nitrocellulose membranes (Fig. 6). Staining with Amido Black revealed a homogenous distribution of proteins over the whole cross section of effective nodules (Fig. 6A). The same was true for ineffective nodules (data not shown). Tissue prints of effective nodules incubated with preimmune serum showed only little

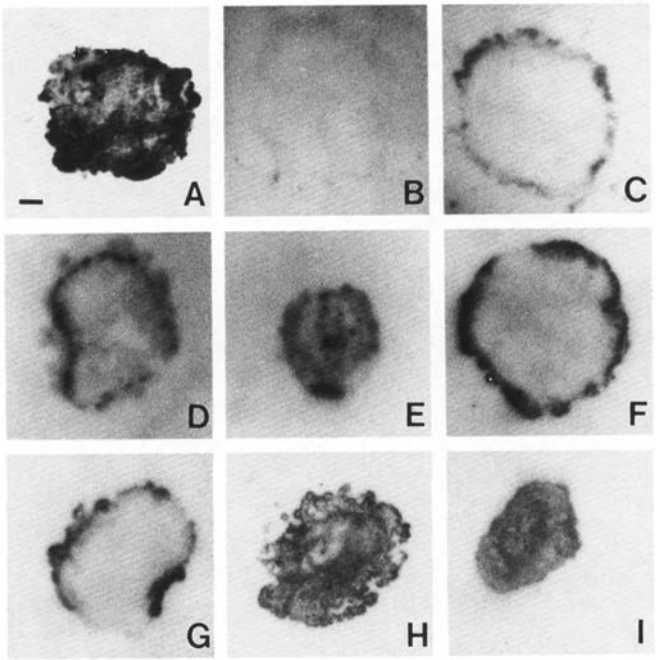

Fig. 6A–I. Immunodetection of chitinase and direct staining for peroxidase in tissue prints of effective and ineffective soybean root nodules. Nodules were induced by *B. japonicum*, strains 61–A–101 (**A–C, F**), RH–31 (**D, G**) or 61–A–24 (**E, H, I**), and harvested 28 d after inoculation. **A** Proteins stained with Amido Black. **B** Immunochemical staining after incubation with preimmune serum. **C–E** Immunochemical staining after incubation with antiserum against bean chitinase. **F–I** Direct staining for peroxidase with 4-chloro-1-naphthol (**F–H**) or 3-amino-9-ethylcarbazole (**I**). Bar = 0.5 mm

background staining (Fig. 6B). Similar results were obtained with tissue prints of ineffective nodules incubated with preimmune serum. The antiserum against bean chitinase revealed the presence of cross-reactive material in the cortex of effective nodules (Fig. 6C) as well as ineffective nodules (Fig. 6D, E). Furthermore, the infected zone of the hypersensitively reacting nodule induced by strain 61–A–24 showed a strong reaction as well (Fig. 6E). Peroxidase activity had a similar distribution, as shown by direct staining of the tissue prints, using 4-chloro-1-naphthol (Fig. 6F, G, H) or 3-amino-9-ethyl carbazole (Fig. 6I) as substrate: peroxidase activity was located mainly in the cortex of effective nodules (Fig. 6F) and in ineffective nodules induced by strain RH–31 (Fig. 6G), but it was present throughout the nodule in ineffective, hypersensitively reacting nodules induced by strain 61–A–24 (Fig. 6H, I).

Discussion

Both chitinase and peroxidase activities are induced in soybean nodules. Chitinase activity has a low constitutive activity in roots which is insensitive to the antiserum against bean chitinase. The cortex of nodules induced by *B. japonicum* strains 61–A–101 and RH–31 has an elevated chitinase activity, and most of it is inhibited by the antiserum against bean chitinase. These data indicate that a chitinase isoenzyme resembling bean chitinase is strongly induced in the nodule cortex regardless of whether the nodule bacteria are fixing nitrogen or not. According to our immunological characterization, this activity resembles the chitinase activity in soybean seeds, characterized previously by Wadsworth and Zikakis (1984). Although this chitinase is not a nodulin in the sense of the definition of a protein exclusively localized in nodules (Legocki and Verma 1980), it should be considered a "nodule-stimulated protein". For peroxidase, two isoenzymes are induced in nodules. This is reminiscent of a recent report describing nodule peroxidase isoenzymes with different specificities for indole-3-acetic acid and syringaldazine (De Forchetti and Tigier 1990). The more slowly migrating isoenzyme is probably the peroxidase (M_r 48 000) described by Puppo et al. (1980). The more rapidly migrating band probably corresponds to the ascorbate peroxidase isoenzyme previously characterized by Dalton et al. (1987), for which they estimated an M_r of 30 000 by SDS–PAGE. The presence of a pseudoperoxidase activity due to leghemoglobin (Puppo et al. 1980) can be excluded in our case since both isoenzymes were also detected in the nodule cortex known to be free of leghemoglobin.

The cortex of nodules has several functions, e.g. as an oxygen-diffusion barrier (Layzell and Hunt 1990). Our work shows that both chitinase and peroxidase are elevated in the cortex of both effective and ineffective nodules. If both enzyme activities can be taken as paradigms of defense reactions, this may indicate that one function of the nodule cortex is to protect the nitrogen-fixing and protein-rich central infected zone from external pathogens.

In the nodules induced by the ineffective strain 61–A–24, chitinase and peroxidase activities were elevated also in the infected zone. This result cannot be sufficiently explained by the high percentage of vacuolized, uninfected cells in this tissue (for pictures see Werner et al. 1980). It is more probable that a hypersensitive reaction is induced, since the interaction of this bacterial strain with soybean roots is characterized also by phytoalexin accumulation and by the early loss of the peribacteroid membranes in nodules (Werner et al. 1985). Chitinase and peroxidase could be considered here as possible weapons against the parasitic bacterium. It may even be that this defense is quite direct since the prototype for the chitinase studied, bean chitinase, has considerable lysozyme activity (Boller et al. 1983). Studies are in progress to examine the possible inhibitory effect of chitinase on growth of *Bradyrhizobium* strains. The induction of peroxidase in nodules induced by strain 61–A–24 might also be seen in the context of pathogen defense and, in particular, of pathogen-dependent induction of lignification as described for many host-pathogen relationships (Svalheim and Robertsen 1990).

With regard to chitinase induction in nodules, a different aspect is worth mentioning. The chemical signals secreted by rhizobia, the so-called Nod factors, are derivatives of chitooligosaccharides (Lerouge et al. 1990; Spaink et al. 1991). It is tempting to speculate that plant

chitinases might hydrolyze Nod factors and thereby interfere with recognition. We are currently testing this possibility.

In conclusion, we suggest a double role for the defense-related enzyme activities in the nodules, i.e. protection of the infected zone from external pathogens in the effective symbiosis and protection of the root system from pathogenic rhizobia in the ineffective symbiosis. In addition, chitinase might also interact with the Nod signals and thereby influence the signalling between the plant and its symbionts.

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