Molecular Plant-Microbe Interactions

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On the cover: Tobacco leaf showing the hypersensitive response elicited by *Pseudomonas syringae* pv. *syringae* 61, *Erwinia amylovora* Ea321, and *E. chrysanthemi* PelABCE$^-$ Out$^-$ mutants. For the article by Bauer *et al.*, see page 573.

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Plant Defense Responses of Host Plants with Determinate Nodules Induced by EPS-Defective *exoB* Mutants of *Bradyrhizobium japonicum*

Martin Parniske, Petra E. Schmidt, Kerstin Kosch, and Peter Müller

Fachbereich Biologie der Philipps-Universität, Karl-von-Frisch-Straße, D-35043 Marburg/Lahn, Germany
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The symbiotic phenotype of *exoB* mutants ΔP5 and ΔP22 of *Bradyrhizobium japonicum 110spc4* was analyzed on the host plants *Glycine max* and *G. soja*. The extent of the symbiotic defects was host dependent. In combination with *G. max*, the *B. japonicum exoB* mutants induced the formation of effective nodules. Infection threads were found in the central nodule tissue of developing nodules, similar to wild-type infected nodules. However, in early stages of the interaction between the mutants and *G. max*, plant defense reactions occurred, among which phytoalexin accumulation was the earliest effect observed. Later the rhizodermis was disrupted by longitudinal cracks caused by cortical cell proliferations, and rhizodermal strips were frequently peeled off the growing nodules. Our results indicate that the intact EPS of *B. japonicum* is necessary for the prevention of plant defense reactions during early interaction with soybean. Combinations between *G. max* and *B. japonicum exoB* mutants seemed to be impaired only transiently, since they resulted in effective nodule formation. However, enhanced concentrations of chitinase within the central nodule tissue of *B. japonicum exoB* mutant induced *G. max* nodules proved the occurrence of plant defense reactions also in later steps of nodule development. On *G. soja*, *B. japonicum exoB* mutants lost their infectivity and induced the formation of white, uninfected and ineffective nodulelike structures at the base of lateral roots.

Additional keyword: glyceollin.

Nitrogen-fixing root nodules are the result of a complex molecular communication process during the symbiotic interaction of rhizobia and legumes. Depending on the host plant species, various types of nodules are formed that can be subdivided into two principal groups. Determinate nodules are spherical, and the central meristematic activity of plant nodule cells stops at a certain predetermined developmental stage. In contrast, indeterminate nodules are cylindrical and have a persistent apical meristem. Apart from the ability to induce meristematic activity in the host root, rhizobia have evolved mechanisms to infect plant cells. The work of several laboratories has unambiguously shown that specific rhizobial exopolysaccharide (EPS) structures are essential prerequisites for a successful infection of indeterminate nodule type legumes, e.g., *Leucaena, Medicago, Pisum, Trifolium,* or *Vicia* species by rhizobia (Borthakur *et al.* 1986; Chakravorty *et al.* 1982; Diebold and Noel 1989; Hotter and Scott 1991; Leigh *et al.* 1987; Müller *et al.* 1988; for a recent review see Leigh and Coplin 1992). EPS mutants of the corresponding microsymbionts *R. loti, R. meliloti,* or *R. leguminosarum* induce the formation of uninfected nodulelike structures that are devoid of bacteroids. Increasing evidence suggests that EPS are involved in the prevention of plant defense responses (Ahlborn and Werner 1991; Pühler *et al.* 1991; Niehaus *et al.* 1993).

In determinate nodule-type legumes such as soybean, the role of EPS is less clear. Rhizobial strains, which could effectively nodulate both determinate and indeterminate types of host plants, were of particular interest to address this question. For instance, EPS^- mutants of the broad host range *Rhizobium* sp. NGR234 formed defective nodules on the indeterminate nodulating host *Leucaena* but formed normal, effective nodules on several tropical legumes of the determinate nodulating type (Chen *et al.* 1985). Hotter and Scott (1991) reported on Exo^- mutants of *Rhizobium loti* which formed defective nodules on *Leucaena* but normal nodules on *Lotus*. The conclusion drawn from these analyses was that EPS is not essential for the formation of effective nodules on determinate nodulating legumes. However, as previously shown, at least in symbiosis with the determinate nodulating legume soybean, rhizobial EPS is not without function. By the construction of specific and genetically defined EPS mutants of *B. japonicum*, it was demonstrated that the elimination of the *exoB* gene of *B. japonicum* leads to specific mutants that form an altered EPS, whereas LPS remained intact. These mutants nodulated soybeans with a delay and a concomitant dramatic loss of competition (Parniske *et al.* 1993). This symbiotic phenotype pointed to a significant function of EPS in the early stages of the symbiotic interaction. Here a more detailed analysis of the symbiotic phenotype of *B. japonicum exoB* mutants raised the question for the specific function of EPS in the infection process of determinate nodules.

The severity of the aberrant phenotypes depended on the host plant. On *Glycine soja*, *B. japonicum exoB* mutants induced meristematic activity but nodule infection was blocked. On *G. max*, several plant defense reactions were observed. In spite of these, the mutants succeeded in reaching the central

Correspondence to: P. Müller.

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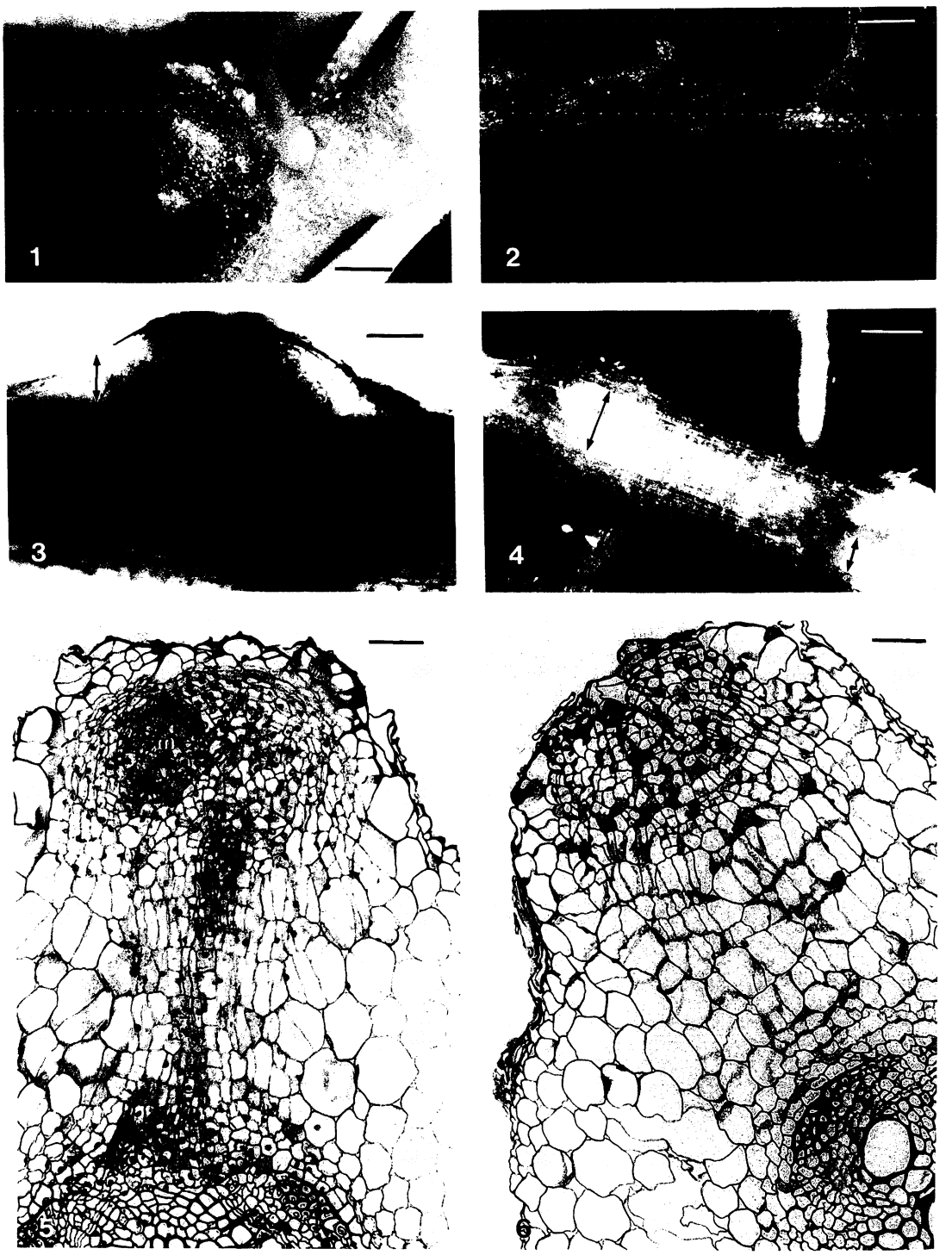


Figs. 1–6. 1, Effective nodule of *Glycine soja* PI468397 induced by *Bradyrhizobium japonicum* 110*spc*4, 20 dpi. × 10, bar = 1 mm. **2,** Nodulelike structures of *G. soja* PI468397 induced by *B. japonicum* ΔP22, 20 dpi. × 5, bar = 2 mm. **3,** Nodule of *G. max* 'Preston' induced by *B. japonicum* ΔP5, 20 dpi. Lateral view. Typically, the outer cell layers separate from the underlying cortex tissue very early in development and are peeled off as the nodule expands (indicated by a bidirectional arrow).×10, bar = 1 mm. **4,** Nodules of *G. max* 'Preston' induced by *B. japonicum* ΔP5, 20 dpi. View from the top. Longitudinal cracks in the rhizodermis frequently occur (bidirectional arrows).×7.5, bar = 1.5 mm. **5,** Micrograph of a longitudinal section through a *G. max* 'Preston' nodule induced by *B. japonicum* 110*spc*4, 15 dpi. Note that the meristem (m) is shielded from direct contact with the environment by non-meristematic, continuous layers of cortical cells. Bar = 50 μm. **6,** Micrograph of a longitudinal section through a *G. max* 'Preston' nodule primordium induced by *B. japonicum* ΔP5, 15 dpi. The lack of the cell layers at the tip of the primordium, normally enclosing the central meristem in a wild type induced nodule (Fig. 5), is apparent. Bar = 50 μm.

nodule tissue via infection threads to establish an effective symbiosis. Nodule formation, however, occurred somewhat delayed. The observations are summarized in a working hypothesis for the biological role of *B. japonicum* EPS in the infection process with determinate type host plants.

RESULTS

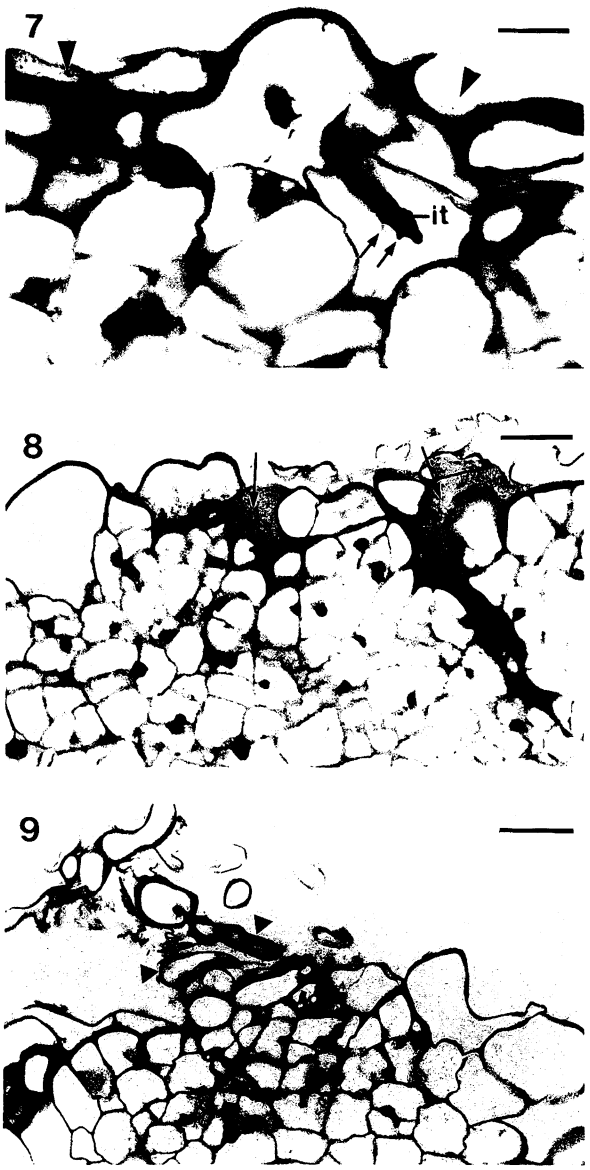
Morphological analysis of soybean nodules induced by *exoB* mutants reveals symptoms of plant defense reactions.

The observation that *B. japonicum exoB* mutants nodulated soybean with a delay of approximately 5 days with respect to the wild type, indicated a disturbance in the early stages of the symbiotic interaction (Parniske *et al.* 1993). The symbiotic phenotype of these mutants was analyzed in more detail to elucidate the characteristics of the mutant phenotype. Since the intensity of plant defense reactions has been shown to depend on the plant species (Parniske *et al.* 1990), two different plant species, *Glycine max* and *G. soja,* were tested for their interaction with *B. japonicum exoB* mutants. In Figures 1–4, wild-type and mutant-induced nodules 20 dpi on *G. soja* (Figs. 1 and 2) and mutant induced nodules on *G. max* (Figs. 3 and 4) are shown. On *G. max*, younger wild-type nodules were surrounded by an intact epidermis which was *in continuum* with the rhizodermis. This outer cell layer was further differentiated in later developmental stages, resulting in the formation of lenticels at the periphery of the emerging nodules. Longitudinal cracks in the rhizodermis appeared only in the vicinity of old nodules and were similar to the local cracks around the sites of emerging lateral roots.

The morphology of nodules induced by *B. japonicum exoB* mutants ΔP5 or ΔP22 on soybean (*G. max*) appeared to be identical and could be macroscopically distinguished from wild-type nodules. Mutant nodules exhibited unusual but characteristic features, differing greatly from one nodule to the next. As shown in Figures 3 and 4, the rhizodermis exhibited longitudinal cracks around the emerging *G. max* nodules induced by the mutant strains. The rhizodermis and adjacent cell layers were ruptured and stripped off due to the expansion of the growing nodule primordium. Occasionally, the cortical cells of the developing nodule were visible as undifferentiated, loosely associated outgrowths of the root, which were apparently uninfected at that stage (Fig. 4). As a consequence of the loss of the outer cell layers, and in contrast to wild-type nodules (Fig. 5), the cortex of mutant nodules was directly exposed to the environment (Fig. 6). Therefore, the nodule cortical cells came in direct contact with the bacterial inoculum (Fig. 7). Remnants of collapsed cells were occasionally found at the outermost distal parts of the cortical tissue (Figs 8 and 9). The outermost cell layer of this tissue had thickened cell walls. Obviously these cells functionally substituted for the regular rhizodermis. Locally restricted cell death of rhizodermal cells and cell wall thickenings of cortical cells were possibly symptoms of structural plant defense responses involving only the outermost cell layers.

Phytoalexin accumulation in soybean root exudate induced by *B. japonicum exoB* mutants.

Since the morphological analysis of soybean nodules induced by *B. japonicum exoB* mutants revealed symptoms of plant defense responses, we analyzed this interaction for the occurrence of other features usually associated with plant defense response. For example, when soybean roots are challenged with phytopathogenic organisms such as *Phytophthora megasperma*, the production of phytoalexins, antimicrobial low molecular weight compounds, is induced (Schmidt *et al.* 1992). The major phytoalexin in soybean is the isoflavonoid glyceollin. As reported earlier (Schmidt *et al.* 1992)



Figs. 7–9. 7, Micrograph of a semithin section through a *G. max* 'Preston' nodule induced by *B. japonicum* ΔP5, 15 dpi. Unusual protuberances (small arrows) of an infection thread (it) are visible which are not observed at parts of the infection threads which have already penetrated more proximal cell layers (not shown). At the periphery, cells of the bacterial inoculum are visible (arrow head). Bar = 10 μm. **8,** Micrograph of a semithin section through a *G. max* 'Preston' nodule primordium induced by *B. japonicum* ΔP5, 15 dpi, showing the accumulation of slime material at the tip of the primordium (arrow). Atypical cracks filled with this material extending into the zone of meristematic activity are visible. Bar = 10 μm. **9,** Micrograph of a semithin section through a *G. max* 'Preston' nodule primordium induced by *B. japonicum* ΔP5, 15 dpi. Remnants of the outer cell layers in the vicinity of the growing primordium are indicated by an arrowhead. Bar = 10 μm.

glyceollin accumulation reaches its maximal value as early as 10 hr after inoculation with *B. japonicum* wild-type strain 110*spc*4, but the concentration was found to be at a considerably lower level compared to elicitation by *P. megasperma*.

The concentration of glyceollin was analyzed during the interaction of soybean with EPS mutants of *B. japonicum*. When soybean (*G. max*) seedlings were axenically incubated in MES buffer, only neglible amounts of glyceollin were detectable in root exudate. In the presence of the *B. japonicum exoB* mutants ΔP5 or ΔP22, enhanced glyceollin concentrations were found in the soybean root exudate as early as after 18 hr of coincubation. Phytoalexin accumulation increased with longer incubation times. After 72 hr, a concentration of glyceollin about ten times higher was measured in the root exudate compared to incubation with the parent strain *B. japonicum* 110*spc*4 (Fig. 10). The observation that glyceollin production increased within the first day after inoculation with *B. japonicum exoB* mutants shows that an early plant defense reaction has occurred.

Immunolocalization of chitinase in the infected zone of soybean nodules induced by *B. japonicum exoB* mutant ΔP22.

Nodules of *G. max* at 21 dpi with *B. japonicum* 110*spc*4 or *B. japonicum exoB* mutant ΔP22 were analyzed for the presence of further symptoms of plant defense reactions in more advanced steps of nodulation. Tissue prints of nodules on nitrocellulose membrane were probed against a bean chitinase antiserum. In wild-type infected nodules, cross-reactive material against bean chitinase antibodies was detectable in the cortex (Fig. 11A). In contrast, in nodules resulting from infection with *B. japonicum exoB* mutant ΔP22 cross-reactive material against bean chitinase was additionally found in the infected zone (Fig. 11B). The reaction was found to be locally restricted. Some of the wild-type nodules exhibited a similar pattern, although less frequently and at lower intensities (data not shown). A quantitation of the effect showed that the reaction occurred in 100% of mutant nodules (46 prints tested) and only in 17% (69 prints tested) of wild-type infected nodules. This was a strong indication that the nodulation defects of the *B. japonicum exoB* mutant not only affected the early steps of infection but that defense-related reactions occurred also in some later stages of nodule development.

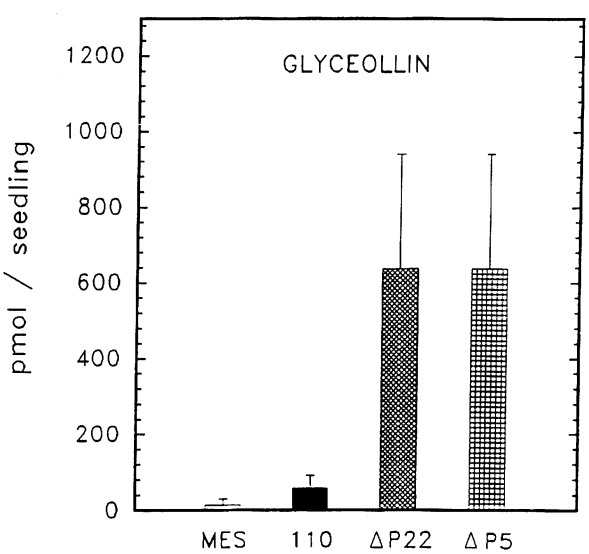


Fig. 10. Exuded amounts of glyceollin by single seedlings of *G. max* (cv. Preston) 72 hr after incubation in MES-buffer (control), 10^8 cfu/plant *B. japonicum* 110*spc*4 (wt), ΔP22 or ΔP5 (EPS mutants). Each value represents the mean of six seedlings. Vertical bars indicate SEM values.

Blocked infection of *Glycine soja* PI468397 by *exoB* mutants.

It is known that the induction of plant defense responses in already developed nodules depends on the specific combination of an inoculant strain and the host plant genotype (Parniske *et al.* 1990). *Glycine soja* PI468397, inoculated with *B. japonicum* 110*spc*4 and grown in petri dishes, had formed four to five effective nodules per plant at 20 dpi. An effective nodule is shown in Figure 1. At the same time, several noninfected nodulelike structures were observed next to the effective nodules on the same root systems. The observation of small, white, ineffective nodules indicated that the combination between *G. soja* and *B. japonicum* strain 110*spc*4 was not an optimal one. On this suboptimal host plant, *G. soja* PI468397, the aberrant nature of the symbiotic phenotype of *B. japonicum exoB* mutants was even more pronounced, compared to the optimal interaction with *G. max*. Numerous, small, white nodulelike structures were observed which frequently were located at the base of lateral roots. Figure 2 shows a part of the root with several lateral roots and a number of such nodulelike structures.

No nitrogenase activity, as indicated by the acetylene reduction assay, was detectable in *G. soja* plants inoculated with *B. japonicum exoB* mutants. Following surface sterilization, no rhizobia could be reisolated from the small white nodules, indicating that they were not colonized by the mutant strains. As found with *G. max* exclusively in combination with *B. japonicum exoB* mutants, the expanding root nodule primordia of *G. soja*, following inoculation with *B. japonicum* wild-type or *exoB* mutant, lead to longitudinal rupturing of the rhizodermis (Fig. 2). In certain instances, after a prolonged incubation time (4–5 wk) in Leonard jars, when plants were already suffering severely from nitrogen deficiency, as indicated by yellow leaves and poor growth, effective nodules occasionally developed on *G. soja* plants infected with *B. japonicum exoB* mutants. Reisolation of the bacteria from these nodules confirmed that all colonies arising on agar plates without antibiotics (kanamycin) were also resistant against kanamycin as expected for *B. japonicum exoB* mutants, and no kanamycin-sensitive colonies (wild-type strain) were obtained. These observations indicated that the nodulelike structures normally observed in this combination, occasionally were infected in more advanced developmental stages, but these events have to be regarded as the rare exception rather than the rule.

DISCUSSION

The construction of *B. japonicum exoB* mutants that produce a structurally modified, galactose-free EPS but unaltered LPS has previously been reported (Parniske *et al.* 1993). The delayed nodulation phenotype on soybean (*G. max*) and the reduced competitiveness of these *B. japonicum exoB* mutants

indicated that a correct EPS structure is essential during the early stages of the symbiotic interaction. The present study attempted to define more precisely the effects of a defective EPS produced by genetically defined *B. japonicum exoB* mutants on the development of determinate nodules. Morphological analyses of soybean nodules induced by *B. japonicum exoB* mutants revealed several characteristic features. Single collapsed plant cells in the outermost cell layers of soybean roots and nodule primordia were found to be typical for this interaction. Massive thickenings of root cell walls in direct contact with the inoculant mutant strains frequently occurred. Furthermore, about 10 times higher concentrations of the phytoalexin glyceollin were detected in the rhizosphere of *G. max* seedlings inoculated with *B. japonicum exoB* mutants, compared to the wild type. Glyceollin accumulation was already detectable as early as after 18 hr of coincubation. This illustrates that this particular event is a very early reaction of the host root. Phytoalexin production, combined with massive cell wall thickening and localized cell death are typical features of the hypersensitive response, a strategy evolved in plants to prevent infection by pathogenic organisms (Lamb *et al.* 1989; Dixon and Lamb 1990).

In their analysis of early morphological events during soybean nodule development, Calvert *et al.* (1984) observed rhizobia-induced cell divisions in rhizodermal cells. Our observations indicated that the soybean rhizodermal cells responded to inoculation with *B. japonicum exoB* mutants in a different way. Instead of dividing, the rhizodermal cells showed symptoms of defense reactions. Apparently, defense responses and cell proliferation did not occur concomitantly. The inability of the outer cell layers to differentiate according to the program in normal nodule development might explain why the rhizodermis was separated from the inner root cortex by the growing nodule primordium.

The defense response of *G. max* to EPS mutants of *B. japonicum* appeared to be locally restricted to plant cells that were in direct contact with the bacterial inoculant. Furthermore, since the *B. japonicum exoB* mutants were ultimately able to colonize the central nodule tissue, the plant defense reactions prevented the inoculated EPS mutants from invading the soybean roots not absolutely but only transiently. As soon as infection threads were formed, nodule development proceeded and a nitrogen-fixing symbiosis was established. From this observation the conclusion can be drawn that the wild-type EPS structure is not required for infection thread formation. However, infection threads in mutant induced nodules often had atypical protrusions (Fig. 7) which might be symptoms of a disturbed interaction in this stage of nodule development.

The detection of plant chitinases in the central nodule tissue in *G. max* nodules infected with *B. japonicum exoB* mutant ΔP22 gives another proof for plant defense reactions in advanced steps of nodule development (21 dpi). Obviously, the altered composition of bradyrhizobial carbohydrate surface compounds is detected by the plant, in the infected and differentiated plant tissue. Therefore, a role of bacterial EPS also in later stages of the development of determinate soybean nodules has to be postulated. Plant chitinases often are associated with plant defense reactions (Boller 1988). Furthermore, chitinases have been reported to inhibit the growth of certain fungi (Schlumbaum *et al.* 1986) and often have lysozyme activity as well, indicating that they may function in defense reactions directed against bacteria (Boller 1988). Enhanced chitinase levels in transgenic plants have been demonstrated to reduce the damage caused by pathogens (Broglie *et al.* 1991). In another experimental approach by Sitrit *et al.* (1993), the expression of *Serratia marcescens* chitinase gene in *Rhizobium meliloti* during symbiosis on alfalfa roots suggested an increased plant resistance to pathogens. The bacterial type of chitinases is related in its sequence to class V of tobacco chitinases which has been cloned and sequenced by Melchers *et al.* (1994). In the infected zone of the *Glycine max* 'Preston' nodules, chitinase was also found in the central nodule tissue of ineffective nodules colonized by bacteria unable to fix nitrogen symbiotically (Staehelin *et al.* 1992).

In a recent review about plant chitinases, Collinge *et al.* (1993) have outlined that chitinase expression is also under developmental control in certain organs and tissues and seems to have an important function in additional non-defensive roles. Of particular interest is the observation that chitinase can inactivate the chitinlike nodulation factors produced by *Rhizobium* and *Bradyrhizobium* strains (Roche *et al.* 1991). Cytochemical and immunological characterization of abortive infections during the *Rhizobium meliloti*-alfalfa symbiotic interaction have lead to the idea that acidic chitinases which accumulate in cells with a hypersensitive reaction, could spe-

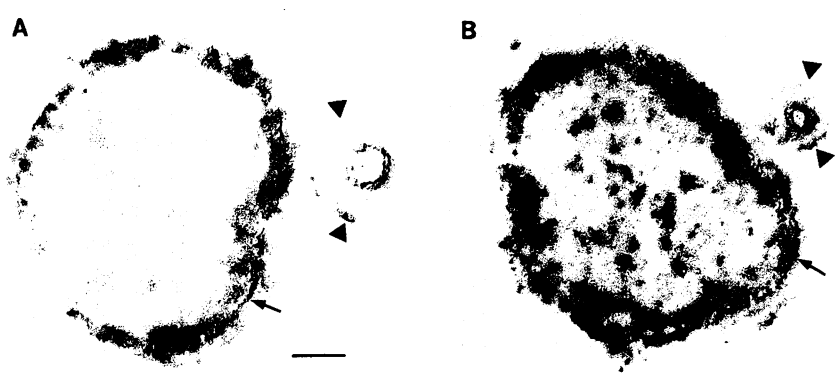


Fig. 11. Immunodetection of chitinase in tissue prints of *G. max* (cv. Preston) nodules 21 dpi with **A**, *B. japonicum* 110*spc*4 and, **B**, *B. japonicum exoB* mutant ΔP22. Arrowheads indicate the rhizodermis in cross sections of the plant root where the nodule originated from. Arrows point to the sclerenchymatic cell layers in the nodule cortex. Bar = 0.3 mm.

cifically hydrolyze Nod factors (Vasse *et al.* 1993). The finding that structural modifications in *Rhizobium meliloti* nodulation factors influence their stability against root chitinases have supported the idea that the action of chitinases may determine the activity of rhizobial nodulation factors (Staehelin *et al.* 1994).

When *B. japonicum exoB* mutants were inoculated onto *G. soja* seedlings, small white nodules were formed which were not colonized by the bacteria. This shows that the severity of the phenotype depended on the host plant species. The uninfected nodules of *G. soja*, induced by *B. japonicum exoB* mutants had some features in common with the aberrant nodules of *G. max*. In both cases the outer cell layers were found to separate from the underlying cortex tissue, suggesting the induction of similar plant defense responses in both plant species. On *G. soja*, the symbiotic interaction of the *B. japonicum exoB* mutants appeared to be disturbed most pronouncedly, since the mutants did not invade the central nodule tissue. However, one has to take into account that *G. soja* PI468397 is only poorly nodulated by *B. japonicum* strains (Parniske *et al.* 1990). This particular plant genotype appears to be better adapted to fast-growing soybean rhizobia, *R. fredii* (Keyser and Cregan 1984).

As previously shown, plant defense responses may also occur in more advanced stages of soybean nodule development. This phenomenon was found in very restricted, specific combinations of host cultivar and *B. japonicum* strain. For example, specific strains of *B. japonicum* induced a hypersensitive response in the nodules of *G. soja* PI468397, but not in nodules of *G. max* (Parniske *et al.* 1990). A similar observation was made in *G. max* nodules infected with a *nifA*-mutant of *B. japonicum* (Parniske *et al.* 1991b) or specific isolates of *B. japonicum* (Werner *et al.* 1985). Depending on the host/strain combination, plant defense responses to rhizobial infection are observed in very early or in later stages of nodule development. Furthermore, in well-adapted combinations of host plant and invading microsymbionts, e.g., *G. max*/*B. japonicum* or *M. sativa*/*R. meliloti*, basic plant defense reactions like glyceollin (Schmidt *et al.* 1992) or medicarpin accumulation (Dakora *et al.* 1993) were observed at low levels. These processes seem to be regulated by fine-tuning mechanisms as recently shown by Vasse *et al.* (1993). They demonstrated that abortive infections of *R. meliloti* on alfalfa were accompanied by the accumulation of phenolic compounds and defense-related proteins in single affected cortex cells. (Brady)rhizobial surface carbohydrates like EPS or LPS appear to be involved in the well-balanced communication processes between the symbiotic partners. Defective EPS- or LPS-structures might result in enhanced plant defense responses.

At different steps of nodule organogenesis, the host plant can rapidly switch to defense responses, suggesting that there exist more than one control mechanism discriminating between a symbiotic and a parasitic type of development. The rhizobia, on the other side, have evolved strategies to overcome these plant defensive barriers. For instance, soybean rhizobia have been shown to possess an inducible resistance against glyceollin (Parniske *et al.* 1991a). Another example for rhizobial strategies to overcome plant defense responses might be the inhibition of plant enzymes by rhizobial EPS, as has been reported by Ahlborn and Werner (1991). They found that the callose synthesizing enzyme (glucan-synthase II) from *G. max* and *Pisum sativum* is specifically inhibited by the addition of wild-type rhizobial exopolysaccharide preparations, whereas surface polysaccarides of different non-rhizobial origins did not perform inhibitory effects.

Recently, Niehaus *et al.* (1993) reported on plant defense and delayed infection of alfalfa nodules induced by a *R. meliloti exoY* mutant. Although this refers to an indeterminate nodule type and, despite the fact that the mutation was within another gene which putatively encodes for a membrane associated protein homologous to hexose transferases (Müller *et al.* 1993), in both systems delayed infections and various indications for plant defense reactions were observed due to defects in the synthesis of extracellular polysaccharides of the inoculant strains. In the combination *G. max* and *B. japonicum exoB* mutant ΔP22 these effects were less pronounced compared to the combinations of *M. sativa*/*R. meliloti exoY* mutant or *G. soja*/*B. japonicum exoB* mutant. Furthermore, in both systems, after a prolonged incubation time, the EPS-deficient mutants were able to infect the plant tissue and to evoke the formation of effective nodules. The use of other host plants like *Macroptilium atropurpureum* in combination with the *B. japonicum exoB* mutant ΔP22 resulted in the formation of necrotic areas within the central nodule tissue (K. Kosch, unpublished).

Based on our observations we propose that intact EPS of *B. japonicum* is necessary to prevent elicitation of plant defense responses in soybean root cells. This working hypothesis explains why *B. japonicum exoB* mutants with a defective EPS were less competitive (Parniske *et al.* 1993) and induced early defense responses in the prospective host plant. Specific rhizobial EPS structures appear to be involved in preventing plant defense responses, at least in the early stages of the symbiosis. Once the bacteria are enclosed by the infection thread, they lose most of their EPS coat and downregulate their EPS production (Tully and Terry 1985). In this situation, the outer membrane is no longer masked by capsular polysaccharides and contact between rhizobial LPS and the plant plasma membrane is thought to occur (Kijne 1992). LPS most probably substitutes for EPS with respect to suppression of plant defense reactions at this stage of the interaction. This idea is consistent with the crucial role of intact LPS in the infection process of soybean reported by Stacey *et al.* (1991). They found that specific LPS mutants of *B. japonicum* were unable to infect the host cells. Further evidence is presented by the work of Perotto *et al.* (1994) about lipopolysaccharide-defective mutants of *R. leguminosarum* which induce a host defense response in pea nodules. As a result, cell and tissue invasion is reduced. The mechanisms by which EPS suppresses defense responses are still unknown. Basically there are two possibilities. The wild-type EPS might prevent plant defense reactions or alternatively, a structurally modified EPS produced by an EPS-defective (brady)rhizobial strain could elicit plant defense reactions. This working hypothesis implies the possibility that, as in *R. meliloti,* the biological function of EPS can be associated with a single active low molecular weight fraction of the entire EPS (Battisti *et al.* 1992). In this respect, the results of K. Miller *et al.* (1994) that cyclic β–1,6-1,3 glucans from *B. japonicum* USDA 110 elicit glyceollin and daidzein production in soybean cotyledons are of special interest. The focus of our future investigations

will be an attempt to answer these questions. This should lead to a better understanding of the prerequisites for the infection of plants not only by symbiotic but also by pathogenic bacteria.

MATERIALS AND METHODS

exoB mutants of *B. japonicum*.

The strain *B. japonicum* 110*spc*4 was used as the wild-type strain (Hahn and Hennecke 1984). The construction of *B. japonicum exoB* mutants was as described previously (Parniske *et al.* 1993).

Growth of plants.

G. max 'Preston' (Pioneer Hi-Bred International Inc., IA) was grown in growth pouches or Leonard jars as previously described (Parniske *et al.* 1993). *G. soja* PI468397 was surface sterilized by immersion in 30% H_2O_2 for 10 min, washed 10 times with sterile water, imbibed with water for 4 hr, and germinated on LN-agar for 3 days in a growth cabinet (for conditions see Parniske *et al.* 1993). The seedlings were transferred to petri dishes (15 cm diameter) containing nitrogen-free nutrient solution (LN-agar, Broughton and Dilworth 1971), solidified with 8 g l^{-1} gelrite (Fa. Roth, Karlsruhe, Germany). For infection of the seedlings, the surface of the gelrite-plates were streaked with 0.5 ml of a suspension of *B. japonicum* cells (approx. 10^7 cfu·ml^{-1}) grown in SMM (Schmidt *et al.* 1992).

Light microscopy.

For light microscopy, specimens were fixed in 2% glutaraldehyde buffered with 50 mM potassium phosphate (KPP), pH 7, for 2 hr, washed twice with KPP, followed by an increasing ethanol series of 25, 50, 75, and 96% ethanol (1 hr each). Embedding was performed in 50% LR White (London Resin Co. Ltd., UK) for 16 hr followed by 100% LR White for at least 4 days with one change of resin. The resin was polymerized at room temperature by the addition of 1% (v/v) accelerator. Thin sections (1–3 μm) were cut using a microtome equipped with a glass knife. Sections were stained with toluidine blue and observed by bright field microscopy.

Analysis of glyceollin.

For the analysis of soybean seedling root exudate, *G. max* 'Preston' seeds were surface sterilized for 10 min in 30% H_2O_2, washed 10 times with sterile H_2O and then soaked for 6 hr in sterile H_2O. Seedlings were germinated on LN-agar for 2 days at 28° C in the dark. Roots of 2-day-old soybean seedlings were transferred to sterile 2.3-ml test tubes containing cellulose acetate filter strips (0.5 × 6.5 cm, Schleicher & Schüll, Göttingen, Germany), submerged in either morpholino ethane sulfonic acid (MES) buffer (5 mM, pH 6.2) or suspensions of the bacterial strains being tested in the same buffer. The seedlings were incubated for 72 hr at 25° C, 13 Wm^{-2}, 75% humidity and a day:night regime of 14:10 hr. The exuded flavonoids adsorbed to the filter strips. After the incubation period, the filters were removed from the test tubes and the flavonoids were extracted twice from the filters with methanol. Separation of daidzein, coumestrol, genistein, and glyceollin was performed by high-performance thin layer chromatography (HPTLC) at −18° C on silica plates (SIL_{60}, 10 × 10 cm, Macherey & Nagel, Düren, Germany) using toluol, ethylacetate, methanol (70/25/5 by vol.) as the mobile phase. To achieve an improved resolution, plates and solvent were precooled to −18° C for 1 hr prior to chromatography. Peaks were identified and quantified by the use of a Desaga densitometer at 285 nm. The concentration of glyceollin was determined using standards of defined concentrations.

Tissue prints and immunological detection of chitinase.

Nodules were cut into halves with a razor blade and directly blotted on a nitrocellulose membrane (Cassab and Varner 1987). For detection of proteins blots where stained with 0.2% (w/v) Ponceau S in 3% (w/v) TCA and 3% (w/v) sulphosalicyclic acid prior to immunodetection. Immunodetection was done according to Day *et al.* (1989). The blot was blocked overnight in blocking buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% [v/v] Tween-20, 5% [w/v] nonfat dry milk), and incubated over night with antiserum against chitinase (1:1,500, Vögeli *et al.*, 1988) in reaction buffer (blocking buffer + 0.1% [w/v] SDS, + 1% [v/v] Triton X-100). After washing 5 times 5 min with wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% [v/v] Tween-20), the membranes were incubated for 2 hr with goat anti-rabbit IgG antibody coupled to alkaline phosphatase (Serva, Heidelberg, FRG; using the dilution recommended by the supplier) in reaction buffer. The membranes were washed 5 times with wash buffer and stained with 5-bromo-4-chloro-3-indolyl-phosphate Na_2-salt (Serva) and Nitro Blue Tetrazolium Na-salt according to Harlow and Lane (1988).

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LITERATURE CITED

Ahlborn, B., and Werner, D. 1991. Inhibition of 1,3-β-glucan synthase from *Glycine max* and *Pisum sativum* by exopolysaccharides of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*. Physiol. Mol. Plant Pathol. 39:299-307.

Battisti, L., Lara, J. C., and Leigh, J. A. 1992. A specific oligosaccharide form of the *Rhizobium meliloti* exopolysaccharide promotes nodule invasion in alfalfa. Proc. Natl. Acad. Sci. USA 89:5625-5629.

Boller, T. 1988. Ethylene and the regulation of antifungal hydrolases in plants. Pages 145-174 in: Oxford Surveys of Plant Molecular and Cell Biology, Vol. 5. B. J. Miflin, ed. Oxford University Press, Oxford.

Borthakur, D., Barber, C. E., Lamb, J. W., Daniels, M. J., Downie, J. A., and Johnston, A. W. B. 1986. A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* and is corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas*. Mol. Gen. Genet. 203:320-323.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Broglie, R. 1991. Transgenic plants with enhanced resistence to the fungal pathogen *Rhizoctonia solani*. Science 254:1194-1197.

Broughton and Dilworth 1971. Control of leghemoglobin synthesis in snake beans. Biochem. J. 125:1075-1080.

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., and Bauer, W.

D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. 62:2375-2384.

Cassab, G., and Varner, J. 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J. Cell Biol. 105:2581-2588.

Chakravorty, A. K., Zurkowski, W., Shine, J., and Rolfe, B. G. 1982. Symbiotic nitrogen fixation: Molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J. Mol. Appl. Genet. 1:585-596.

Chen, H., Batley, M., Redmond, J., and Rolfe, B. G. 1985. Alteration of the effective nodulation properties of a fastgrowing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis. J. Plant Physiol. 120:331-349.

Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U., and Vad, K. 1993. Plant chitinases. Plant J. 3:31-40.

Dakora, F. D., Joseph, C. M., and Phillips, D. A. 1993. Alfalfa (*Medicago sativa* L.) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. Plant Physiol. 101:819-824.

Day, D. A., Price, D. G., and Udvardi, M. K. 1989. Membrane interface of the *Bradyrhizobium japonicum-Glycine max* symbiosis: Peribacteroid units from soybean nodules. Aust. J. Plant Physiol. 16:69-84.

Diebold, R., and Noel, K. D. 1989. *Rhizobium leguminosarum* exopolysaccharide mutants: Biochemical and genetic analyses and symbiotic behaviour on three hosts. J. Bacteriol. 171:4821-4830.

Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:339-367.

Hahn, M., and Hennecke, H. 1984. Localized mutagenesis in *Rhizobium japonicum*. Mol. Gen. Genet. 193:46-52.

Harlow, E., and Lane, D. 1988. Antibodies, a Laboratorial Manual. Cold Spring Habor Laboratory, Cold Spring Harbor, NY.

Hotter, G. S., and Scott, D. B. 1991. Exopolysaccharide mutants of *Rhizobium loti* are fully effective on a determinate nodulating host but are ineffective on an indeterminate nodulating host. J. Bacteriol. 173:851-859.

Keyser, H. H., and Cregan, P. B. 1984. Interactions of selected *Glycine soja* Sieb. & Zucc. genotypes with fast- and slow-growing soybean rhizobia. Crop Sci. 24:1059-1062.

Kijne, J. W. 1992. The *Rhizobium* infection process. Pages 349-398 in: Biological Nitrogen Fixation. G. Stacey, R. H. Burris, and H. Evans, eds. Chapman & Hall, New York.

Lamb, C. J., Lawton, M. A., Dron, M., and Dixon, R. A. 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56:215-224.

Leigh, J. A, Reed, J. W., Hanks, J. F., Hirsch, A. M., and Walker, G. C. 1987. *Rhizobium meliloti* mutants that fail to succinylate their calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell 51:579-587.

Leigh, J. A., and Coplin, D. L. 1992. Exopolysaccharides in plant-bacterial interactions. Annu. Rev. Microbiol. 46:307-346.

Melchers, L. S., Apotheker-de Groot, M., van der Knaap, J. A., Ponstein, A. S., Sela-Buurlage, M. B., Bol, J. F., Cornelissen, B. J. C., van den Elzen, P. J. M., and Linthorst, H. J. M. 1994. A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. Plant J. 5:469-480.

Miller, K. J., Hadley, J. A., and Gustine, D. L 1994. Cyclic β-1,6-1,3 glucans of *Bradyrhizobium japonicum* USDA 110 elicit isoflavonoid production in the soybean (*Glycine max*) host. Plant Physiol. 104:917-923.

Müller, P., Hynes, M., Kapp, D., Niehaus, K., and Pühler, A. 1988. Two classes of *Rhizobium meliloti* infection mutants differ in exopolysaccharide production and coinoculation properties with nodulation mutants. Mol. Gen. Genet. 211:17-26.

Müller, P., Keller, M., Weng, W. M., Quandt, J., Arnold, W., and Pühler, A. 1993. Genetic analysis of the *Rhizobium meliloti exoYFQ* Operon: ExoY is homologous to sugar transferases and ExoQ represents a transmembrane protein. Mol. Plant-Microbe Interact. 6:55-65.

Niehaus, K., Kapp, D., and Pühler, A. 1993. Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS I)-deficient *Rhizobium meliloti* mutant. Planta 190:415-425.

Parniske, M., Ahlborn, B., and Werner, D. 1991a. Isoflavonoid-inducible resistance to the phytoalexin glyceollin in soybean rhizobia. J. Bacteriol. 173:3432-3439.

Parniske, M., Fischer, H. M., Hennecke, H., and Werner, D. 1991b. Accumulation of the phytoalexin glyceollin I in soybean nodules infected by a *Bradyrhizobium japonicum nifA* mutant. Z. Naturforsch. 46c:318-320.

Parniske, M., Kosch, K., Werner, D., and Müller, P. 1993. *ExoB* mutants of *Bradyrhizobium japonicum* with reduced competitivity on *Glycine max*. Mol. Plant-Microbe Interact. 6:99-106.

Parniske, M., Zimmermann, C., Cregan P. B., and Werner, D. 1990. Hypersensitive reaction of nodule cells in the *Glycine* sp./*Bradyrhizobium japonicum*-symbiosis occurs at the genotype-specific level. Bot. Acta 103:143-148.

Perotto, S., Brewin, N. J., and Kannenberg, E. L. 1994. Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. Mol. Plant-Microbe Interact. 7:99-112.

Pühler, A., Arnold, W., Buendia-Claveria, A., Kapp, D., Keller, M., Niehaus, K., Quandt, J., Roxlau, A., and Weng, W. M. 1991. The role of the *Rhizobium meliloti* exopolysaccharides EPS I and EPS II in the infection process of alfalfa nodules. Pages 189-194 in: H. Hennecke and D. P. S. Verma, eds. Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 1. Kluwer Academic, Dordrecht.

Roche, P., Lerouge, P., Ponthus, C., and Promé, J. C. 1991. Structural determination of bacterial nodulation factors involved in the *Rhizobium meliloti*-alfalfa symbiosis. J. Biol. Chem. 266:10933-10940.

Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324:365-367.

Schmidt, P. E., Parniske, M., and Werner, D. 1992. Production of the phytoalexin glyceollin I by soybean roots in response to symbiotic and pathogenic infection. Bot. Acta 105:18-25.

Sitrit, Y., Barak, Z., Kapulnik, Y., Oppenheim, A. B., and Chet I. 1993. Expression of *Serratia marcescens* chitinase gene in *Rhizobium meliloti* during symbiosis on alfalfa roots. Mol. Plant-Microbe Interact. 6:293-298.

Stacey, G., So, J.-S., Roth, L. E., Bhagya Lakshmi, S. K., and Carlson, R. W. 1991. A lipopolysaccharide mutant of *Bradyrhizobium japonicum* that uncouples plant from bacterial differentiation. Mol. Plant-Microbe Interact. 4:332-340.

Staehelin, C., Müller, J., Mellor, R. B., Wiemken, A., and Boller, T. 1992. Chitinase and peroxidase in effective (Fix^+) and ineffective (Fix^-) soybean nodules. Planta 187:295-300.

Staehelin, C., Schultze, M., Kondorosi, E., Mellor, R. B., Boller, T., and Kondorosi, A. 1994. Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. Plant J. 5:319-330.

Tully, R. E., and Terry, M. E. 1985. Decreased exopolysaccaride synthesis by anaerobic and symbiotic cells of *Bradyrhizobium japonicum*. Plant Physiol. 79:445-450.

Vasse, J., de Billy, F., and Truchet, G. 1993. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. Plant J. 4:555-566.

Vögeli, U., Meins, F., Jr., and Boller, T. 1988. Co-ordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta 174:364-372.

Werner, D., Mellor, R. B., Hahn, M. G., and Grisebach, H. 1985. Soybean root response to symbiotic infection. Glyceollin I accumulation in an ineffective type of soybean nodules with an early loss of the peribacteroid membrane. Z. Naturforsch. 40c:179-181.