The Impairment of the Nodulation Process, Induced by a *Bradyrhizobium japonicum* Exopolysaccharide Mutant is Determined by the Genotype of the Host Plant

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The deletion mutant *Bradyrhizobium japonicum* ΔP22 produces a structurally altered exopolysaccharide. The nodulation of two cultivars each of *Glycine max* and *Glycine soja,* and cultivars of *Macroptilium atropurpureum* and *Vigna radiata,* infected with this mutant was examined in order to analyze the role of the exopolysaccharide in the infection process of plants with a determinate nodule type. All host plants analyzed exhibited delayed nodulation and formed fewer nodules per plant. The extent of the impairments depended on the genotype of the host plant. Morphological studies confirmed these results. In *V. radiata* later steps in nodule development proceeded without further disturbances, whereas with *G. soja* PI 407287 minor changes were detected. In contrast, the inoculation of *G. soja* PI 468397 and *M. atropurpureum* lead to the formation of nodules most of which were not infected by *Bradyrhizobium japonicum* ΔP22 (Inf⁻). However, on *M. atropurpureum* at least some effective nitrogen-fixing nodules developed. Such nodules did not emerge from *G. soja* PI 468397. Inf⁻ nodules were arrested in an early stage of nodule development, and symptoms of plant defense responses were observed.

Introduction

Inoculation of legumes with gram-negative soil bacteria of the genera *Rhizobium/Bradyrhizobium* and *Azorhizobium* leads to the formation of a new symbiosis-specific organ, the root nodule. The nodule provides a microaerobic habitat where the bacteria are able to fix atmospheric dinitrogen. The development of this symbiosis requires a bi-directional molecular communication. In the early stage of this interaction the host plant exudes flavonoid compounds which stimulate the expression of the *nod* genes (Peters *et al.,* 1986). The *nod* genes in turn encode enzymes involved in synthesis of the nod factors, lipooligosaccharide signal molecules that cause symbiosis-specific changes in the plant root (Lerouge *et al.,* 1990; Fisher and Long, 1992; Göttfert, 1993).

In search of other signal molecules that play a significant role in the symbiotic interaction surface polysaccharides like exopolysaccharides (EPS) and lipopolysaccharides (LPS) have been found to be essential for the establishment of an effective symbiosis (Finan *et al.,* 1985; Leigh *et al.,* 1987; Müller *et al.,* 1988; Stacey *et al.,* 1991). Inoculation of alfalfa which produces indeterminate nodules with *Rhizobium meliloti* EPS mutants results in the induction of nodule-like organs; nevertheless, the central nodule tissue is not invaded by the bacteria (Inf⁻ phenotype). A similar phenotype was observed with *B. japonicum* LPS mutants in combination with plants of the determinate nodule type (Stacey *et al.,* 1991). However, the inoculation of the latter plants with EPS mutants leads to the establishment of an effective nitrogen-fixing symbiosis, indicating that intact EPS is not essential for the symbiosis with determinate type legumes (Diebold and Noel, 1989; Hotter and Scott, 1991).

As previously reported the nodulation of *G. max* "Preston" and *G. soja* PI 468397 is impaired in an early developmental stage when in-

oculated with the exopolysaccharide mutant *B. japonicum* ΔP22, which produces an EPS of an altered structure. Furthermore, plant defense reactions were detected (Parniske *et al.*, 1993; Parniske *et al.*, 1994). The present report shows that inoculation with this EPS mutant can lead to an Inf⁻ phenotype on determinate nodule type plants and that the degree of the impairment mainly depends on the genotype of the host plant.

Materials and Methods

Plant material and inoculum strains

Glycine max "Preston" and "Maple Arrow" seeds were obtained from Pioneer Hi-bred Inc., U.S.A. and Ottawa Research Station, Canada, respectively. *Glycine soja* PI468397 and PI407287 seeds were a gift from P. B. Cregan, Beltsville, U.S.A. *Macroptilium atropurpureum* seeds were generously given by S. Crank, Wye College, London, U.K. *Vigna radiata* seeds were obtained from a local source.

The wild type strain was *Bradyrhizobium japonicum* 110 spc 4 (Regensburger and Hennecke, 1983). The construction of the mutant strain *Bradyrhizobium japonicum* ΔP22 was described elsewhere (Parniske *et al.*, 1993). Rhizobial strains were grown at 28 °C in a mineral medium with succinate (SMM) as the sole carbon source (Schmidt *et al.*, 1992).

Plant growth and inoculation

Seeds were surface-sterilized by immersion in 30% H_2O_2 for 15 min, washed ten times with sterile water, imbibed 6 h in sterile water and washed again. Seeds were pregerminated for 3 days at 28 °C in the dark on nitrogen-free nutrient agar (Werner *et al.*, 1975). Seeds of *G. soja* PI468397 were scarified prior to germination. After 3 days the seedling roots were submersed in a suspension containing 10^6 bacteria×ml⁻¹ for 30 min and were then transferred into growth pouches with 10^6 bacteria per plant in 20 ml. The location of the root tip was marked. The plants were grown in a growth chamber at a constant temperature of 25 °C, 75% relative humidity, and a day/night regime of 16/8 h with a light intensity of 2550 $\mu E \times m^{-2} \times s^{-1}$. Every two days 20 ml nitrogen-free nutrient solution were added. At 21 dpi, the plants were harvested.

Acetylene reduction assay

Acetylene reduction assays of cut root systems in glass tubes were performed according to Koch and Evans (1966).

Fixation, embedding and microtomy of the nodules and staining of the sections

For embedding in L. R. White (London Resin Co.) nodules were harvested 21 days after inoculation, cut with a razor blade and fixed in 2% glutaraldehyde in 50 mM potassium phosphate buffer, pH 6.8, for 1 or 2 h under vacuum. After washing in buffer the nodules were dehydrated in a graded ethanol series, left over night in L. R. White:ethanol 1:1 (v/v), and finally infiltrated in L. R. White. Polymerization was performed with 1% enhancer at room temperature. Semithin sections of about 1 μm were cut with an OM U2 Reichert microtome equipped with a Ralph glass-knife. Sections were stained for 90 sec at 60 °C with Toluidineblue O (1%, w/v, Toluidineblue O 0.1%, w/v, Na-borate in distilled water) and rinsed with warm tap water. The slides were viewed by light microscopy (Orthoplan, Leitz) and for the occurrence of autofluorescence with a Leitz Laborlux fluorescence microscope (excitation filter 270–380 nm, barrier filter 510–580 nm).

Results

Delay of nodulation

The organogenesis of leguminous root nodules follows a certain developmental program under defined cultural conditions. Less adapted combinations of host plant and inoculum strains result in a delayed onset of nodulation, which has also been found for deletion mutants of *Bradyrhizobium japonicum* defective in EPS synthesis (Parniske *et al.*, 1993). Inoculation with *B. japonicum* ΔP22 led to a delay of nodule development for all host plants tested. Visible nodule primordia appeared three to four days later than on wild type-inoculated plants. Infection of rhizobia in roots of leguminous host plants occurs *via* young or just developing root hairs which are located in a short distance above the root tip at the time of inoculation (Bhuvaneswari *et al.*, 1980). Therefore, a quantitative method to determine the time course of nodule induction is to measure the distance of

the uppermost nodule to the root tip of the main root at the time of inoculation. The uppermost nodule induced by the wild type *B. japonicum* 110 *spc* 4 is located just above or below the root tip mark (Fig. 1). The distance from the uppermost nodule to the root tip mark (rtm) was greater in all cases when the EPS-deficient mutant *B. japonicum* ΔP22 was inoculated to the plants (Fig. 1). This effect was most pronounced with *Vigna radiata*. No nodules developed at the main root, although fewer nodules on the main root of this host plant were generally induced by the wild type (Table I). The phenomenon of impaired nodulation was further confined by the observation that in all plant species tested, the number of plants nodulated at the main roots was reduced and the proportion of nodules at the main root was decreased.

Inoculation of *Glycine soja* PI 468397 with *B. japonicum* ΔP22 resulted in the formation of tissue protrusions which almost exclusively emerged at the sites of lateral root development. These were not regarded as nodules because macroscopically they resembled undifferentiated cell clusters.

Reduced nodule number per plant and effectivity of the mutant nodules

Another indication for the disturbed nodulation process following the inoculation with *B. japonicum* ΔP22 was the reduced total number of nodules per plant compared to wild type-infected plants (Fig. 2). Again the most pronounced phenotype was observed with *Vigna radiata* and the non-infected *G. soja* PI 468397, whilst *G. soja* PI 407287 was less affected. The decrease in the total number of nodules per plant was greater in *G. max* "Maple Arrow" than with "Preston".

In order to analyze the effectiveness of the symbiosis with *B. japonicum* ΔP22, specific nitrogenase activity was measured by the acetylene reduction assay (Fig. 3). Mutant and wild type-
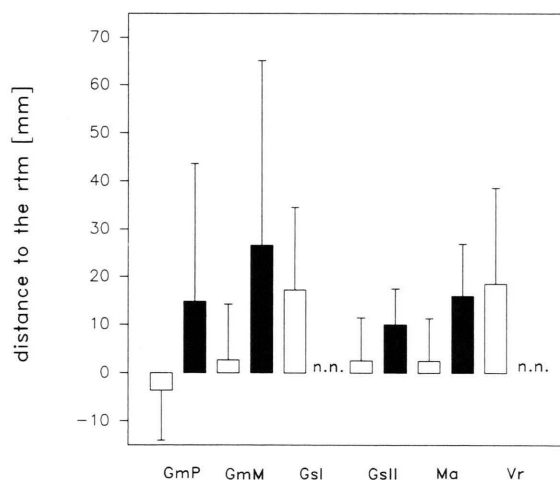

Fig. 1. Distance of the uppermost nodule to the root tip mark (rtm), of the main root at the time of inoculation of different host plants inoculated with *B. japonicum* 110 *spc* 4 (white bars) and *B. japonicum* ΔP22 (black bars). Negative values indicate a position above the rtm directed to the shoot, positive values a position below the rtm directed to the root tip. The data represent the average (with standard deviation) of three independent experiments (T-test, number of plants $n > 17$, $p < 0.05$). Differences were significant for all host plants (GmP, *Glycine max* "Preston"; GmM, *Glycine max* "Maple Arrow"; GsI, *Glycine soja* PI 468397; GsII, *Glycine soja* PI 407287; Ma, *Macroptilium atropurpureum*; Vr, *Vigna radiata*; n.n. no nodules at the main root. The tissue protrusions of *G. soja* PI 468397 were not regarded as nodules in these experiments).
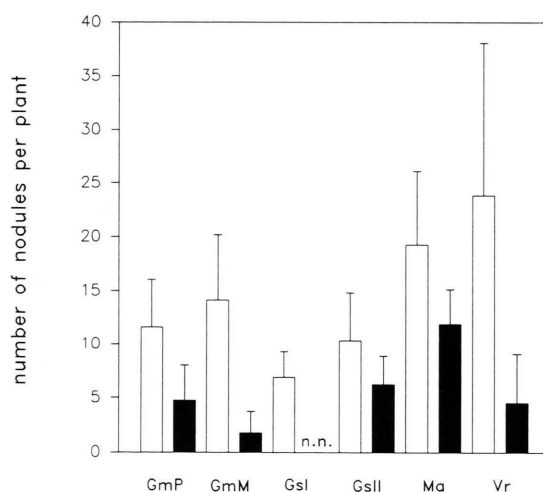
Fig. 2. Number of nodules per plant inoculated with *B. japonicum* 110 *spc* 4 (white bars) and *B. japonicum* ΔP22 (black bars). The data represent the average (with standard deviation) of three independent experiments (T-test, $n > 17$, $p < 0.05$). Differences between plants inoculated with *B. japonicum* 110 *spc* 4 and with *B. japonicum* ΔP22 were significant for all host plants (GmP, *Glycine max* "Preston"; GmM, *Glycine max* "Maple Arrow"; GsI, *Glycine soja* PI 468397; GsII, *Glycine soja* PI 407287; Ma, *Macroptilium atropurpureum*; Vr, *Vigna radiata*; n.n., no nodules. The tissue protrusions of *G. soja* PI 468397 were not regarded as nodules in these experiments).

Table I. Distribution of the nodules of different host plants after inoculation with *B. japonicum* 110*spc*4 and *B. japonicum* ΔP22.

Inoculum	Rate of plants with nodules on the main root in %		Rate of nodules on the main root in %	
	110[a]	ΔP22[b]	110[a]	ΔP22[b]
Host plant				
Glycine max cv. "Preston"	100.0	52.4	46.7	28.4
Glycine max cv. "Maple Arrow"	92.1	18.0	32.4	17.3
Glycine soja PI 468397	95.3	0.0	50.0	0.0
Glycine soja PI 407287	100.0	68.8	100.0	33.3
Macroptilium atropurpureum	94.4	75.0	26.9	11.4
Vigna radiata	32.2	0.0	6.0	0.0

[a] *Bradyrhizobium japonicum* 110*spc*4.
[b] *Bradyrhizobium japonicum* ΔP22.

induced nodules of *V. radiata*, *G. max* "Maple Arrow", and *G. soja* PI 407287 reduced acetylene to a similar degree. Values obtained with the latter two cultivars were slightly increased. However, acetylene reduction of *G. max* "Preston" nodules infected with *B. japonicum* ΔP22 exceeded that of wild type-infected nodules significantly (Fig. 3).
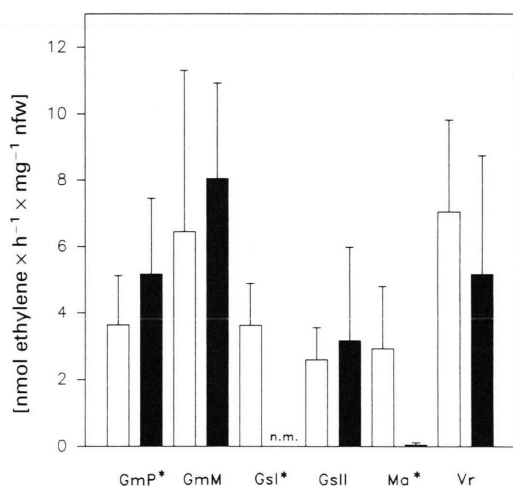

Fig. 3. Amount of reduced acetylene per mg nodule fresh weight (nfw) of root nodules infected with *B. japonicum* 110*spc*4 (white bars) and *B. japonicum* ΔP22 (black bars). The data represent the average (with standard deviation) of three independent experiments (T-test, $n > 11$, $p < 0.05$). Differences between plants inoculated with *B. japonicum* 110*spc*4 and with *B. japonicum* ΔP22 were significant (marked by an asterisk) for *Glycine max* "Preston", *Glycine soja* PI 468397, and *Macroptilium atropurpureum* (GmP, *Glycine max* "Preston"; GmM, *Glycine max* "Maple Arrow" GsI, *Glycine soja* PI 468397; GsII, *Glycine soja* PI 407287; Ma, *Macroptilium atropurpureum*, Vr, *Vigna radiata*; n.m., no acetylene reduction measurable).

In contrast to this, acetylene reduction was hardly measurable with mutant-induced nodules of *Macroptilium atropurpureum* (Fig. 3). This observation could not be explained by the inability of the mutant bacteroids to fix nitrogen, since the identical mutant strain was fully effective in symbiosis with other host plants. However, the nodule cells of this host plant were infected by *B. japonicum* ΔP22 less densely in most of the cases, as will be shown below. Acetylene was not reduced by the cell protrusions of *G. soja* PI 468397 (Fig. 3). This tissue was not infected and no bacteroids could be reisolated.

Histological evidence for a disturbed infection process

Apart from delayed onset and low frequency of nodulation, the infection process in *Vigna radiata* did not differ between inoculation with *B. japonicum* ΔP22 or the wild type *B. japonicum* 110*spc*4. The effective nodules induced by both strains showed the typical determinate nodule type with a comparable colonization of the central nodule tissue. Similar observations were made with *G. soja* PI 407287. However, in one of the examined nodules vacuoles were visible in infected cells 21 dpi (Fig. 4 b), indicating a disturbed symbiotic interaction.

The cell protrusions, developed on *G. soja* PI 468397 after inoculation with *B. japonicum* ΔP22 were not infected. Growth of these tissues mostly arrested at a just visible state. With this host plant no infection proceeded as shown in Fig. 4 a, although remeristematization of cortex
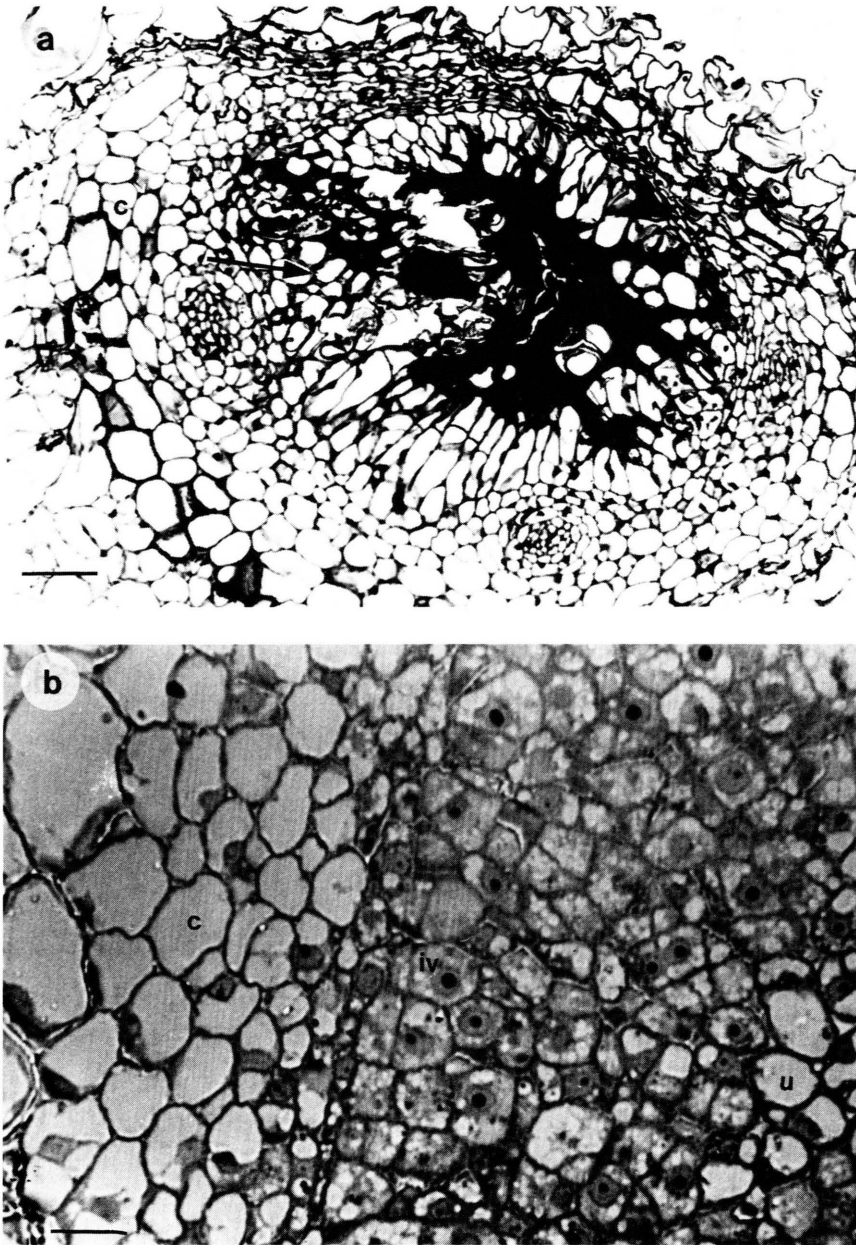
Fig. 4. Thin sections of nodules (21 dpi) of *Glycine soja* PI 468397 (a) and *Glycine soja* PI 407287 (b) induced by *B. japonicum* ΔP 22 (arrow indicates cell wall thickening; iv, infected cell with vacuoles; u, uninfected cell; c, cortex cell; bar a: 35 μm, b: 7 μm).

cells was induced. However, the origin of these cell protrusions as a root nodule primordium was demonstrated by the arrangement of the tissue layers examined *via* cross-sectioning. The cells of the central tissue were unusually elongated. They were surrounded by a cortex tissue including vascular bundles and a ring of sclerenchymatic cells like infected determinate type nodules (Fig. 4 a).

At 21 dpi, the central tissue was already destroyed and filled with a readily dyeable mass of unknown composition. Some of the cell walls of adjacent cells were thickened (Fig. 4 a). The destroyed tissue and the surrounding tissues showed a blue autofluorescence after incitation by UV light indicating the presence of phenolic substances (Kosch, unpublished results).
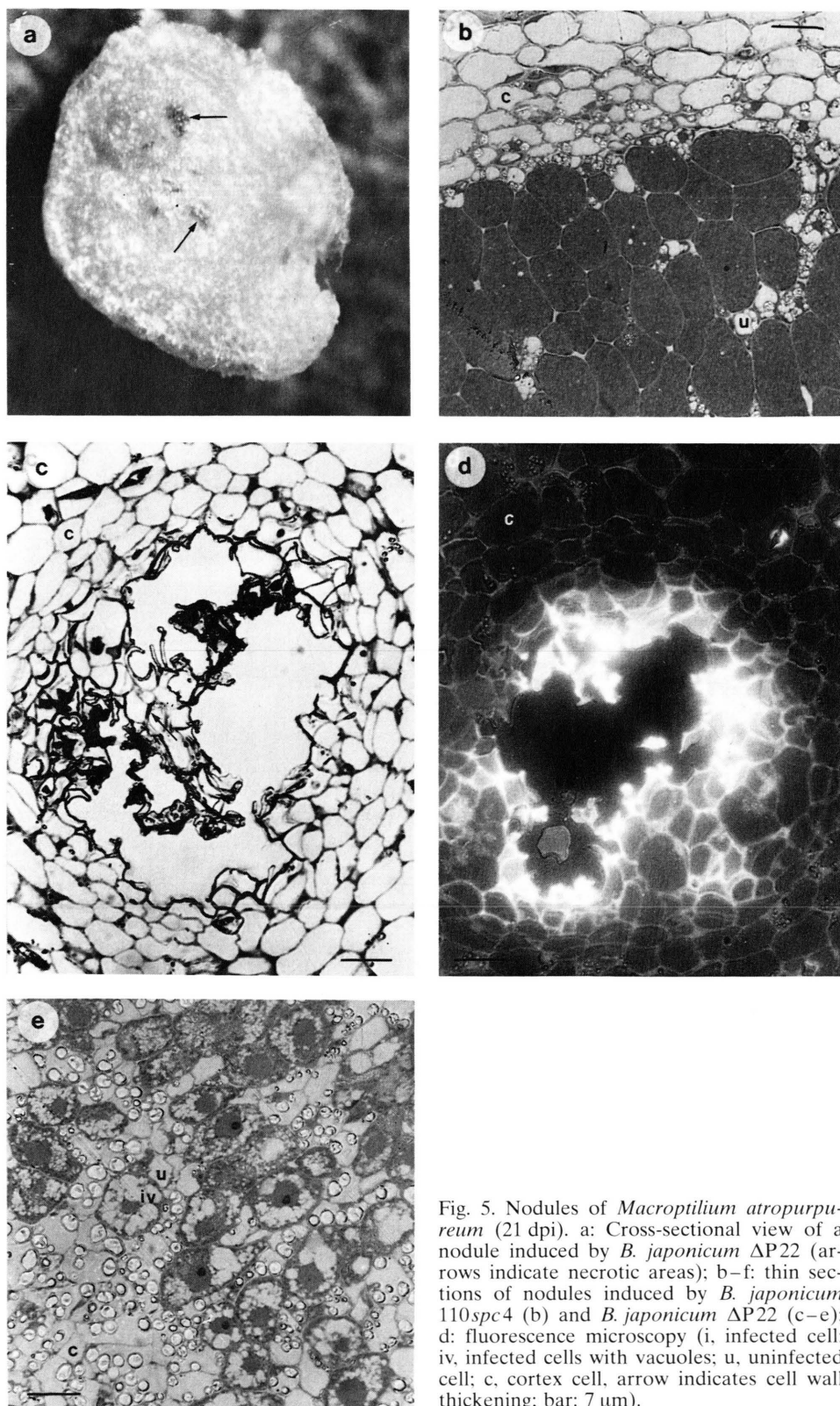
Fig. 5. Nodules of *Macroptilium atropurpu-reum* (21 dpi). a: Cross-sectional view of a nodule induced by *B. japonicum* ΔP22 (arrows indicate necrotic areas); b–f: thin sections of nodules induced by *B. japonicum* 110*spc*4 (b) and *B. japonicum* ΔP22 (c–e); d: fluorescence microscopy (i, infected cell; iv, infected cells with vacuoles; u, uninfected cell; c, cortex cell, arrow indicates cell wall thickening; bar: 7 μm).

The symbiotic phenotype of *M. atropurpureum* inoculated with *B. japonicum* ΔP22 is even more complex. On this host plant, *B. japonicum* ΔP22-induced nodules which resembled macroscopically and microscopically wild type-induced nodules with a red coloured central infected zone (Fig. 5b). However, these nodules represented less than 20% of the total amount of nodules. Other types of nodules with an only slightly red or uncoloured central tissue, indicating little or no leghemoglobin production were found on the same plants. Furthermore, in some of the nodules without any visible traces of leghemoglobin, necrotic areas evolved within the central tissue (Fig. 5a). The cells of the central tissue were small compared to normal infected cells and no prominent nuclei were observed, demonstrating that the cells of these nodules had not undergone the transformation to polyploidy which normally takes place during the infection process (Verma and Long, 1983). The properties of the tissue of these areas impaired thin sectioning. As a consequence most of the tissue was torn out (Fig. 5c). On the border of this tissue, cell wall thickening occurred (Fig. 5c), and a blue fluorescence was detected under UV light (Fig. 5d). However, most of the mutant-induced nodules of *M. atropurpureum* showed a slightly red colour. In these nodules the infected zone was not uniformly distributed *via* the central tissue, but restricted zones of infected tissue were separated by a greater part of uninfected tissue. In addition to this, large vacuoles in the infected cells were observed (Fig. 5e). Vacuolization was also detected in some mutant nodules with a uniformly distributed infected central tissue. The vacuolated cells were not densely packed with bacteroids like wild type cells (Fig. 5b). However, the vacuolated cells contained large nuclei (Fig. 5), indicating that the transformation to polyploidy had taken place. The different types of impairment in *M. atropurpureum* show that the variance of nodule development not only differs with the genotype of the host plant, but also within a single plant.

Discussion

The inoculation with the exopolysaccharide mutant *B. japonicum* ΔP22 has several effects on the nodulation process of various host plants with a determinate nodule type. The first visible indication is the belated appearance of the nodule primordia, which implies a delay of nodule development. This effect is further documented by the greater distance of the uppermost nodule from the root tip mark of mutant inoculated plants and the increased percentage of nodules induced at lateral roots compared to wild type inoculation. Rhizobial strains with a delay in nodulation are not as able to infect root hairs susceptible at the time of inoculation as the wild type, but root hairs which develop after some time of root growth which results in a greater distance in relation to the rtm.

For *Macroptilium atropurpureum* an enhanced level of phenylalanine ammonium lyase, which is involved in the plant defense was detected in seedling roots two days after infection with *B. japonicum* ΔP22 (Kosch, unpublished results). The inoculation of *G. max* "Preston" with *B. japonicum* ΔP22 leads to the accumulation of the phytoalexin glyceollin in the root exudate of seedlings 48–72 h after infection (Parniske *et al.*, 1994). On the other hand, the resistance of *B. japonicum* 110spc4 towards glyceollin can be induced (Parniske *et al.*, 1991; Kape *et al.*, 1992). In this respect the delay of nodulation can possibly be regarded as a period of bacterial adaptation to induced plant defense responses.

The nodule number per plant is governed by the autoregulation system of the plant (Caetano-Anollés and Gresshoff, 1991). The arrest of the developing infection threads which show symptoms of a hypersensitive reaction is proposed to play a role in this respect within wild type-infected alfalfa plants (Vasse *et al.*, 1993). The induced plant defense response after inoculation with *B. japonicum* ΔP22 could block more successful infections. The extent of the effect depends on the degree of the induced plant defense which is manifested in the genotype of the host plant and the capability of the bacteria to cope with the specific defense mechanisms.

Parniske *et al.* (1994) showed that *B. japonicum* ΔP22 infects *G. max* "Preston" in a different mode of entry. Comparable macroscopical symptoms like disruption of the rhizodermis caused by cortical cell proliferations were also observed with *G. max* "Maple Arrow" (Kosch, unpublished results). However, the infection of these host plants and *V. radiata* finally leads to an effective nitrogen-fixing symbiosis, with a regularly infected

zone, suggesting an only transient impairment of nodulation at an early stage of the infection process. Once the mutant bacteria have invaded the host cells further defense reactions have no influence on the effectiveness of the symbiosis. The specific nitrogenase activity of mutant-induced nodules of *G. soja* PI 407287 and the two cultivars of *G. max* even exceeds that of wild type-induced nodules. This increased specific nitrogenase activity could be based on a facilitated oxygen diffusion in these nodules as a result of the former impairment of the nodulation process. An enhanced O_2 partial pressure leads to an increased nitrogenase activity since under normal physiological conditions O_2 tension is suboptimal (Stripf and Werner, 1980; Layzell *et al.*, 1990). As a consequence of the reduced number of nodules per plant, fewer nodules have to accomplish for the nitrogen demand of the host plant, a problem which is met by a higher effectiveness. This can not be achieved in *M. atropurpureum* and *G. soja* PI 468397 where the colonization of the host cell by *B. japonicum* ΔP 22 is partly, respectively completely, blocked by the plant.

The severe disturbation of nodule development of *G. soja* PI 468397 was already described on the macroscopic level (Parniske *et al.*, 1994). The disintegration of the central nodule tissue and the accumulation of phenolic substances suggest a strong defense response of the host plant during the infection process at the stage of nodule growth. This finally leads to the necrosis of the central nodule tissue. The severity of the effect can probably be seen in connection with the reduced compatibility of this plant genotype with the slow growing wild type strains of *B. japonicum* and the preference for fast growing strains (Keyser and Cregan, 1984; Parniske *et al.*, 1990). Thus an already poorly adapted interaction can easier be unbalanced than a well-adapted symbiotic relationship represented by *G. soja* PI 407287 with slow growing strains. With this host plant, vacuolization of infected cells was only detected once. The later impairment of the symbiosis seemed to be rather an exception than the rule.

The interaction between *B. japonicum* ΔP 22 and *G. soja* PI 468397 is the first example of an Inf⁻ phenotype of a plant with a determinate nodule type in combination with an exopolysaccharide mutant and underlines the importance of this surface polysaccharide in symbiosis with these host plants. The phenotype resembles the situation of alfalfa nodules (indeterminate nodule type) induced by exo⁻ mutants of *Rhizobium meliloti*. The induction of the synthesis of phenolic substances as precursors for a reinforcement of cell walls and the dying of tissue is similar to a plant defense response in form of a hypersensitive reaction (Niehaus *et al.*, 1993).

The different nodule types of *M. atropurpureum* induced by *B. japonicum* ΔP 22 demonstrate that the influence on nodule development not only varies between different genotypes but also on a single plant. The reason for this is not yet understood. At the worst, the bacteria are not capable to invade the central nodule tissue and the tissue disintegrates. Possibly the necrotic spots represent the place of release of the bacteria from the infection threads and the altered surface structure provoked further defense responses at this stage. However, no histological proof for this hypothesis could be found. The cells of the central tissue of these nodules had not undergone the transformation to polyploid cells which normally takes place during the infection process (Verma and Long, 1983). The enhanced reaction could therefore be due to a failure of the bacteria to provoke essential physiological changes of the plant cells. In other nodules infected zones were not uniformly distributed *via* the central tissue but zones of infected tissue was separated by a greater part of uninfected tissue. In these nodules release from the infection thread occurred. However, the struggle of the bacteria to cope with the host defense responses seems to slow down the infection process and the growth of the bacteria. As a consequence, the bacteria were possibly not able to keep pace with the cell division activity of the plant, the distribution of the infected cells remained restricted to confined small areas. However, the vacuolated cells contained large nuclei indicating that the transformation to polyploidy had taken place. This raises the question whether polyploidy is a major prerequisite for cell invasion as already suggested by Verma and Long (1983).

Relying on the assumption that the disturbance of nodulation is caused by the induction of defense responses of the host plants, the velocity of the induction seems to determine the degree of the impairment. Although minor defense responses

occurred with *G. max* "Maple Arrow" and "Preston" (Parniske *et al.*, 1994) and *G. soja* PI 407287 at later stages of nodule development, the effectiveness of this symbiosis was not altered, and nitrogen could be fixed. The defense responses of *G. soja* PI 468397 and *M. atropurpureum* resulted in a more pronounced disturbation, up to an Inf⁻ phenotype implicating a lack of nitrogen supply for the host plant which finally leads to nitrogen deficiency symptoms. The former hypothesis that EPS is not essential for plants with determinate nodule type (Gray and Rolfe, 1990) has to be newly reflected. The genotype of plant species determines the reaction of the plant towards EPS mutants which can be very different as shown in this paper. Thus, the hitherto existing studies with EPS mutants may only represent combinations with partners where the disturbance of EPS had no major effects on the effectiveness of the symbiosis (Borthakur *et al.*, 1986; Diebold and Noel, 1989; Hotter and Scott, 1991) as has been shown for *Vigna radiata* in combination with *B. japonicum* ΔP 22.

The altered EPS structure of *B. japonicum* ΔP 22 leads to the reinforced induction of a plant defense while the wild type only induces minor reactions which do not impair the establishment of the symbiotic interaction (Schmidt *et al.*, 1992). How wild type EPS is involved in the reduction of the defense responses and why different plants show a different degree of defense reaction still remain interesting questions to be solved. Further experiments with *B. japonicum* ΔP 22 will hopefully be helpful to answer these questions.

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