Accumulation of the Phytoalexin Glyceollin I in Soybean Nodules Infected by a *Bradyrhizobium japonicum nifA* Mutant

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The mutation of the *nifA* gene of *Bradyrhizobium japonicum* leads to a pathogenic-like response of the host plant. Soybean nodules induced by the *nifA* mutant A 9 exhibited symptoms of a hypersensitive reaction (HR) normally observed in plant pathogen interactions as localized death of infected cells and the accumulation of the phytoalexin glyceollin I. Little or no glyceollin I was present in nodules elicited by wild type *B. japonicum* 110*spc*4.

Bradyrhizobium japonicum fixes nitrogen in symbiotic root nodules of its host plant *Glycine max*. A number of *B. japonicum* genes have been identified that are necessary for the establishment of an effective interaction [1]. Apart from the *nif* and *fix* genes that are directly involved in the process of nitrogen fixation, other genes are required for the specific communication between the two symbiotic partners (for review see [2]).

The *nif* and *fix* genes are under positive control by the regulatory *nifA* gene [3, 4]. Mutations in any of the *nifD*, *nifK*, *nifH*, *nifE*, *nifN*, *nifS*, *nifB*, *fixA*, *fixB*, or *fixC* genes lead to normally developed, though ineffective, nodules [5–8]. In marked contrast, deletion of the regulatory gene *nifA* has a pleiotropic effect on symbiosis: nodules elicited by a *B. japonicum nifA* mutant strain are not only ineffective but also show severe degradation of the nodule tissue. A striking feature of these nodules is their necrotic appearance not later than 20 days after infection [3, 9]. This phenotype suggests that a plant defence response takes place upon infection with the *nifA* mutant strain, *i.e.* the symbiotic interaction appears to have changed to a pathogenic one. It is known that in response to a pathogenic infection soybeans synthesize the phytoalexin glyceollin I [10]. Here we report that soybean nodules formed by *nifA*$^-$ *B. japonicum* induce a similar response.

B. japonicum 110*spc*4 (wild type) and its *nifA*$^-$ derivative A 9 were grown as described [3]. Strain A 9 was constructed by replacing a 838 bp *nifA*-internal *Xho*I fragment by a 2347 bp *Xho*I fragment from transposon Tn*5* encoding the kanamycin resistance gene *aphII*. For details see Fischer *et al.* [3] and Thöny *et al.* [11].

Plant infection tests with *Glycine max* L. Merr. *cv.* Preston and *cv.* Williams were performed as reported previously [5] except that seed surface sterilization was done in 0.7% sodium hypochlorite, and the plants were grown in wide-necked glass jars. On days 12, 15, 18, 21, 24, 27 and 30 after infection all nodules from three plants infected with *B. japonicum* wild type or *nifA* mutant strains were collected separately and used for the determination of glyceollin I contents using a radioimmunoassay as described by Hahn *et al.* [10] modified by [12].

During the sampling period from day 12 to day 30 after infection little or no glyceollin I was detected in nodules formed by *B. japonicum* wild type strain 110*spc*4. By contrast, from day 12 to day 15, glyceollin I concentration in nodules elicited by the *nifA* mutant showed a marked increase to a level of approx. 6 µmol per g nodule dry weight which was maintained until day 30 after infection (Fig. 1). Due to the small size of the nodules no accurate glyceollin I determinations could be performed before day 12. At day 12 glyceollin I concentrations in nodules of the *nifA* mutant exhibited strong variations from plant to plant. Some plants had nodules with no detectable glyceollin I (as in wild type-induced nodules) while others had already accumulated significant levels of the phytoalexin. This indicated that the specific plant defence response against the *B. japonicum* mutant strain was elicited around or shortly before day 12 in the infection process. The cultivars Preston and Williams both responded with glyceollin I accumulation. Cultivar Williams produced a slightly higher amount of glyceollin I than *cv.* Preston (data not shown).

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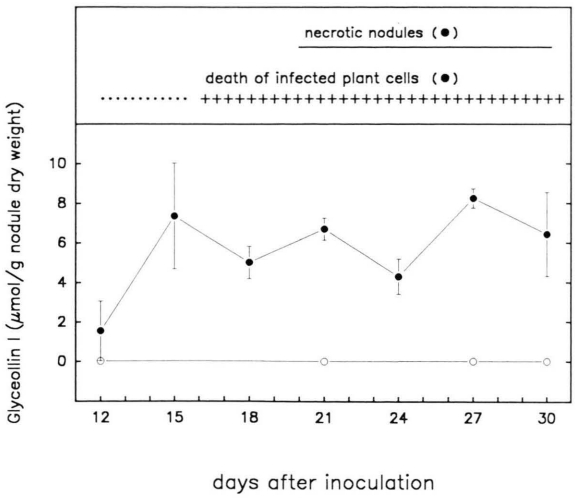

Fig. 1. Development of glyceollin I accumulation in nodules of *G. max cv.* Preston infected with *B. japonicum* wild type 110*spc*4 (○) or *nifA* mutant A 9 (●). In the periods indicated in the upper part of the figure the following observations were made: (——), macroscopically visible necrosis of nodule tissue beginning in the center of the nodules; (·····), simultaneous occurrence of intact and disintegrated infected plant cells as observed by electron microscopy [15]; (+++), predominant occurrence of disintegrated infected plant cells.

Parallel to these experiments we examined the nodule morphology by electron microscopy. We confirmed the previously reported observations by Studer *et al.* [9]. Beginning at day 12 after inoculation, localized death of infected plant cells was indicated by lysis and complete loss of cellular compartmentalization in nodules of the *nifA* mutant. Adjacent, non-infected plant cells did not show symptoms of cell disintegration (data not shown). About eight days after the onset of glyceollin I production, *i.e.* at a later stage of the plant defence response, necrosis of the nodule tissue became visible macroscopically. Nodules with visible necrosis always contained high amounts of phytoalexin.

The two features, phytoalexin accumulation and localized cell death at the infection site, are the main symptoms of a hypersensitive response (HR) described for incompatible plant-pathogen interactions [13]. By these reactions, the soybean response to infection by *B. japonicum* A 9 is indistinguishable from that of an incompatible plant pathogen. Furthermore, the level of glyceollin I observed in nodules of the *nifA* mutant was in the same range as found in the pathogenic interaction of soybeans with the fungus *Phytophtora megasperma* f. sp. *glycinea* [10]. Interestingly, elevated glyceollin I levels have also been observed in ineffective soybean nodules induced by the genetically undefined *B. japonicum* strain 61 A 24 [14].

Djordjevic *et al.* [15] described a HR-like response in root hairs of *Macroptilium atropurpureum* after infection with an adenine-auxotrophic, polysaccharide overproducing Tn*5* mutant of the fast-growing broad-host-range *Rhizobium* sp. NGR 234. This observation, together with the results presented here, suggest that wild type rhizobia have genes and gene products preventing the plant defence response. Depending on the rhizobial mutant strain analyzed, it appears that the defence response is elicited either at an early stage, *i.e.* during the initial infection process, or at a late stage, *i.e.* when nodule formation is already completed as exemplified by *B. japonicum* A 9. Since it is known that *nifA* is involved in the activation of numerous *nif* and *fix* genes [3, 4], induction of a HR in nodules of *B. japonicum* A 9 is probably due to the altered expression of one or several *nifA*-controlled genes rather than to the absence of NifA per se.

In addition, the defence response can be host controlled, because *B. japonicum* strain 123 was shown to form effective nodules with low glyceollin I content on *G. max*, but induced a HR in nodules of *G. soja* PI 468 397 [12].

Finally, it is interesting to note that in *Pseudomonas syringae* pv. *phaseolicola* a regulatory gene (*hrpS*) has been identified which shows homology to the rhizobial *nifA* genes. This gene is required for the pathogenic interaction with the host plant as well as for the induction of a HR on non-host plants [16, 17]. In the symbiotic as well as in the pathogenic plant-bacterium interaction the presence of a functional *nifA* gene or *nifA*-homologue appears to be essential for a compatible interaction.

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