Bradyrhizobium japonicum Mutants Defective in Cyclic β-Glucan Synthesis Show Enhanced Sensitivity to Plant Defense Responses

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Susceptibility of the nitrogen-fixing soybean symbiont *Bradyrhizobium japonicum* to inducible plant defense metabolites such as phytoalexin and H_2O_2, was investigated. On the wild-type strain USDA 110 the soybean phytoalexin, glyceollin, showed bacteriostatic activity. Viable bacteria isolated from intact nodules were adapted to glyceollin. H_2O_2 in physiological concentrations did not affect wild-type bacteria. *B. japonicum* mutants defective in the biosynthesis of cyclic β-(1→3)-(1→6)-glucans showed higher susceptibility to both phytoalexin and H_2O_2.

Introduction

Members of the genus *Bradyrhizobium*, whose ability to participate in symbiotic associations is well known, synthesize high levels of characteristic cyclic β-(1→3)-(1→6)-glucans (Rolin *et al.,* 1992), even within soybean root nodules (Gore and Miller, 1993). These periplasmic oligosaccharides are implicated to function as osmotically-active solutes during hypoosmotic adaptation (Breedveld and Miller, 1998). Additionally, there is a growing body of evidence that cyclic β-glucans play a role during infection of the host plant soybean (*Glycine max* L.) (Dunlap *et al.,* 1996; Mithöfer *et al.*, 1996; Bhagwat *et al.,* 1999). The cyclic β-glucan synthesis locus in *B. japonicum* has been isolated and two genes, *ndvB* and *ndvC*, have been identified (Bhagwat and Keister, 1995; Bhagwat *et al.,* 1996). Mutations within *ndvB* resulted in the absence of cyclic β-glucans (strain AB-14). As described for other *fix*$^-$ strains (Werner *et al.,* 1985), strain AB-14 formed ineffective (i.e., unable to fix nitrogen) but differentiated nodules on soybean (Bhagwat and Keister, 1995) which contained a significant higher concentration of the soybean phytoalexins, glyceollins (Bhagwat *et al.*, 1999). Mutations within the *ndvC* locus (strain AB-1) resulted in the synthesis of cyclodecacis-(1→3)-β-glucosyl (Bhagwat *et al.,* 1999). Strain AB-1 was unable to induce effective nodules and formed very small pseudonodules without viable bacteria, with a delay of nearly two weeks (Bhagwat *et al.,* 1996; Dunlap *et al.,* 1996). These pseudonodules contained around 9-fold higher concentrations of glyceollins compared with non-infected roots (Bhagwat *et al.,* 1999). The glyceollin content of nodules infected with the *B. japonicum* wild-type (strain USDA 110) was in the same range as found for the control using uninoculated roots (Bhagwat *et al.,* 1999). This suggested that the presence of the cyclic β-glucans is important during soybean nodulation. In the present study, we examined the influence of typical plant defense factors, such as phytoalexins or H_2O_2, on free-living cells of the *B. japonicum* mutant strains AB-1 and AB-14, respectively, in comparison to the cyclic β-(1→3)-(1→6)-glucan-containing wild-type strain.

Materials and Methods

Bradyrhizobium japonicum strains (USDA 110, AB-1, AB-14), cyclic β-(1→3)-(1→6)-glucans, and the isoflavonoids 3,6a,9-trihydroxypterocarpan and glyceollin isomers from soybean were available from the laboratory collections. Determination of glyceollin was carried out by high-performance liquid chromatography as described (Mithöfer *et al.,* 1996).

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Bacteria were grown in arabinose-gluconate medium (Cole and Elkan, 1973). To monitor bacterial growth in the presence of phytoalexins, log-phase cells were inoculated into fresh medium in microcultures of 400–500 µl according to Parniske *et al.* (1991). Phytoalexins were added from stock solutions in ethanol. During experiments the maximal ethanol concentration was 1% (v/v) which showed no effect on bacterial growth in control experiments.

To isolate bacteroids from nodules, five to ten nodules were collected and surface-sterilized first with 20% (v/v) industrial bleach solution for 6 min followed by extensive washing with sterile H_2O and then with 70% (v/v) ethanol (for 1 to 2 min), followed by extensive washing with sterile H_2O. Subsequently, the nodules were mechanically crushed with a sterile toothpick and 200 µl grinding buffer (0.1 M potassium phosphate, pH 6.8, 0.1 mM $MgCl_2$, 1 µg ml^{-1} DNase) (Keister and Marsh, 1990) was added. After centrifugation at $1000 \times g$ for 30 sec, the supernatant of that suspension containing the bacteroids was collected and used for further experiments.

For analyzing the effect of H_2O_2, log-phase *B. japonicum* cells were mixed with top agar and poured on arabinose gluconate medium plates (Bhagwat *et al.*, 1999). After 20 h of incubation at 28 °C, 6-mm-Whatman filter paper discs containing various concentrations of H_2O_2 were placed on the top agar as described (Loprasert *et al.*, 1997). The diameter of the cleared zone around the discs was measured after 48 h.

Results and Discussion

To investigate the influence of isoflavonoid phytoalexins such as 3,6a,9-trihydroxypterocarpan (THP) or glyceollin isomers on *B. japonicum* strains their growth behavior was analyzed. Whereas THP, the biosynthetic precursor of glyceollins, had no influence on the growth of *B. japonicum* up to a concentration of 200 µM, the presence of glyceollins at the same concentration of 200 µM prolonged the lag periods of the wild type *B. japonicum* strain USDA 110 and strain AB-1, respectively, about 20 h (Fig. 1). After the cultures reached the logarithmic phase their doubling time was not affected. Although strain AB-1 makes "wrong" β-glucan for symbiosis, this cyclic-β-glucan is sufficient to protect cells against rising defense by the host. In contrast, strain AB-14 was strongly inhibited by glyceollins (Fig. 1). At 200 µM, glyceollins nearly completely inhibited growth, indicating high susceptibility to phytoalexins in comparison with the wild-type strain. Since cyclic glucans have been suggested to form inclusion complexes with molecules such as (iso)flavonoids (Breedveld and Miller, 1998), cyclic β-glucans isolated from USDA 110 were added to the medium at a final concentration of 1 mM. However, this treatment did not complement the mutation to restore the growth of AB-14 in the presence of 200 µM glyceollins indicating that exogenously added cyclic β-glucans were not effective in caging glyceollins (Fig. 1B). Moreover, none of the *B. japonicum* strains analyzed in these experiments metabolized glyceollins, confirming earlier results (Parniske *et al.*, 1991). Why the absence of cyclic β-glucans in *B. japonicum* AB-14

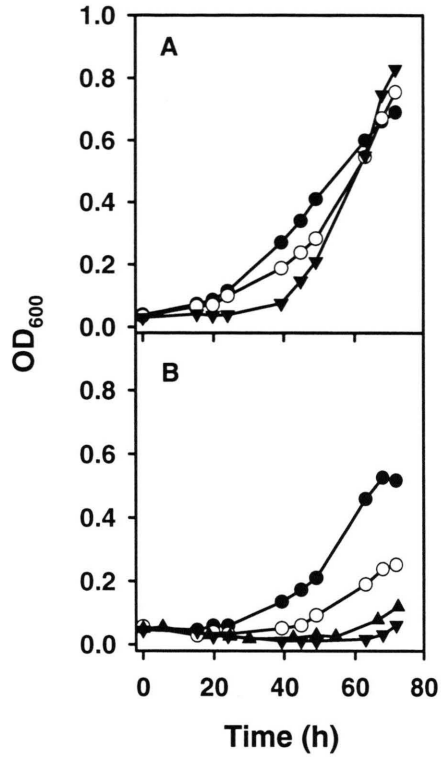

Fig. 1. Growth of *B. japonicum* strains USDA 110 (●), AB-1 (○), and AB-14 (▼) in microcultures in (A) the absence and (B) the presence of 200 µM glyceollins. Strain AB-14 was also supplemented with 1 mM cyclic β-glucans (▲).

caused increased sensitivity of the mutant to glyceollins remains to be investigated.

To analyze the properties of *B. japonicum* cells grown during symbiotic interaction, a method for re-isolation of bacteroids from soybean nodules was established (see Materials and Methods). Viable bacteria could be obtained from nodules formed with strain USDA 110. The growth of these bacteria was not affected by glyceollins even at the highest concentration (200 μM) used (Fig. 2). This suggested that the isolated cells were adapted to glyceollins within the nodule environment as shown for free-living cells by treatment with isoflavones (Parniske *et al.*, 1991). Although glyceollins alone had no strong effect on free-living wild-type cells, a bacteriostatic effect of the phytoalexin on the bacteroids in the nodules can not be ruled out. Bacteroids from AB-14 could be isolated as well but bacteria did not start growing even at 10 μM glyceollins, probably due to their high susceptibility to phytoalexins.

Active plant defense responses against microbes involve increased production of reactive oxygen species (Lamb and Dixon, 1997). To analyze the protection capability of the different *B. japonicum* strains against increasing concentrations of H_2O_2, we quantitatively determined their sensitivity by the disc diffusion killing zone method (Loprasert *et al.*, 1997). Both mutant strains, AB-1 and AB-14, showed a ten-fold higher susceptibility to H_2O_2 than strain USDA 110 (Table I). The concentration necessary to induce clearing zones in case of the mutant strains was 1 mM H_2O_2. However, such a concentration has never been reported in plants challenged by microbes. The highest H_2O_2 concentration detected in soybean treated with *P. sojae* elicitor was around 45 μM (Mithöfer *et al.*, 1997). This is too low to affect the *B. japonicum* strains AB-1 and AB-14 directly, suggesting that the primary role of H_2O_2 in plant defense is not its antimicrobial activity, at least in the interaction between soybean and *B. japonicum*.

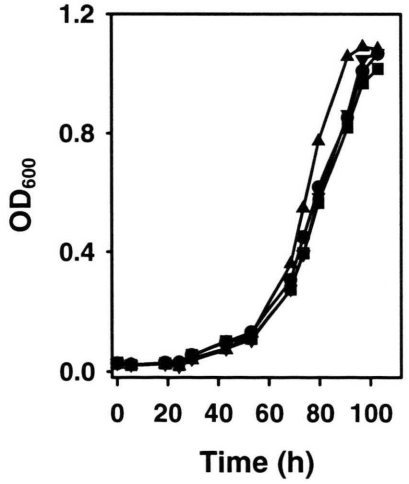

Fig. 2. Growth of *B. japonicum* strain USDA 110, after isolation from intact nodules, in the absence (●) and presence of 2 μM (■), 20 μM (▼), and 200 μM (▲) glyceollins.

Table I. Sensitivity of various *B. japonicum* strains (USDA 110, AB-1, AB-14) to increasing concentrations of H_2O_2.

H_2O_2 [mM]	USDA 110	AB-1	AB-14
	Clearing zone [mm][a]		
0	n.d.[b]	n.d.[b]	n.d.[b]
1	n.d.[b]	6.3 ± 0.6	6.0 ± 0.0
10	5.7 ± 0.6	7.7 ± 0.6	8.7 ± 0.6
100	12.0 ± 1.0	13.3 ± 1.2	23.7 ± 0.6

[a] The results are the mean ± SD from three independent experiments; [b] n.d.: not detected.

The results of this study demonstrate that free-living wild-type *B. japonicum* cells show only low sensitivity against glyceollins or H_2O_2 in their environment. Susceptibility of *B. japonicum* to these plant defense factors correlates with the absence of cyclic β-glucans supporting a role of these carbohydrates during symbiotic interactions with the soybean host.

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