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Site-Directed Mutagenesis of the β-(1→3),β-(1→6)-D-Glucan Synthesis Locus of *Bradyrhizobium japonicum*

Arvind A. Bhagwat[1,2] and Donald L. Keister[1]

[1]Soybean and Alfalfa Research Laboratory, USDA, Agricultural Research Service, Bldg. 011, HH-19, BARC-W, Beltsville, MD 20705 U.S.A.; and [2]Department of Agronomy, University of Maryland, College Park, MD 20742 U.S.A.

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Bradyrhizobium japonicum **produces β-(1→3),β-(1→6)-D-glucans, which appear to be functionally equivalent to β-(1→2)-D-glucans produced by *Rhizobium* and *Agrobacterium* spp. These compounds are involved in osmoregulation and may play an important role in symbiosis and tumorigenesis. We previously isolated a cosmid clone from a *Bradyrhizobium japonicum* USDA 110 gene library coding for proteins involved in the synthesis of β-(1→3),β-(1→6)-D-glucans (Bhagwat et al. FEMS Microbiol. Lett. 114:139-144, 1993). Site-directed Tn*5* mutagenesis was used to delimit the region coding for β-(1→3),β-(1→6)-D-glucan synthesis. The Tn*5* insertion and flanking DNA region was homogenotized into the chromosome of *B. japonicum* USDA 110. A recombinant strain was isolated which was defective in synthesis of β-(1→3),β-(1→6)-D-glucans. The membrane fraction also failed to incorporate UDP-(^{14}C)glucose into soluble, neutral glucans. The mutant was defective in motility and growth under conditions of low osmolarity and formed ineffective nodules on *Glycine max* (soybean). These results indicate that the cyclic β-(1→3),β-(1→6)-D-glucans may be required for an effective symbiosis in *B. japonicum*.**

Additional keywords: membrane-derived oligosaccharides, nodule development, osmoregulation.

Current evidence clearly shows that cyclic β-glucans of the *Rhizobiaceae* function as osmolytes and that they are important during the interaction of these microorganisms with host plants (Cangelosi et al. 1987; Dylan et al. 1990; Breedveld and Miller 1994). Most studies on the role of the cyclic β-glucans have been with *Rhizobium meliloti* and *Agrobacterium tumefaciens* which produce cyclic β-(1→2)-D-glucans (e.g., Cangelosi et al. 1987, 1989; Ielpi et al. 1990). β-Glucans from these organisms are composed of 17 to 25 glucose residues linked solely with β-(1→2)-D-glycosidic bonds and they may be substituted with phosphoglycerol, phosphoethanolamine, and/or succinate (York et al. 1978; Batley et al. 1987; Hisamatsu et al. 1987). The role of the substituents is not known.

Corresponding author: A. A. Bhagwat; E-mail: Arvind@wam.umd.edu

Several other microorganisms that interact with plants, such as *Bradyrhizobium japonicum* and *Azospirillum brasilense,* synthesize glucans linked by β-(1→3),β-(1→6)-D-glycosidic bonds (Dudman and Jones 1980; Miller et al. 1990; Tully et al. 1990; Altabe et al. 1994). While *Azospirillum* glucans are linear molecules, *Bradyrhizobium* glucans are cyclic and composed of 11 to 13 glucosyl residues and may be substituted with phosphocholine (Rolin et al. 1992). Although the glucans are structurally different in *Bradyrhizobium* and *Rhizobium/Agrobacterium* spp., the two glucan species appear to be functional analogs of each other (Miller and Gore 1992; Bhagwat et al. 1993). The homologous *ndv*B (nodule development) and *chv*B (chromosomal virulence) genes of *Rhizobium* and *Agrobacterium,* respectively, code for membrane proteins which appear be intermediates in β-(1→2)-glucan synthesis (Zorreguieta and Ugalde 1986; Ielpi et al. 1990). They are very large, estimated to be 319-kDa by nucleotide sequence analysis (Ielpi et al. 1990) and 235-kDa by SDS-PAGE (Zorreguieta and Ugalde 1986). The proteins become transiently labeled with (^{14}C)glucose (with UDP-[^{14}C]glucose as substrate) and appear to have characteristics of an intermediate in β-(1→2)-glucan synthesis (Zorreguieta and Ugalde 1986) but little is known about the catalytic mechanism. Analysis of the deduced sequence of the *ndv*B gene product revealed no significant homology with other known proteins (Ielpi et al. 1990). Iannino and Ugalde (1993) reported the identification of a 90-kDa inner membrane protein from *B. japonicum* with kinetic properties similar to the 235-kDa protein of *R. meliloti.* However, these observations are yet to be confirmed as Cohen and Miller (1991), and Bhagwat and Keister (1992) were unable to detect such a protein by SDS-PAGE either by protein staining or labeling with (^{14}C)glucose.

There is no hybridization observed between the β-(1→2)-glucan synthesis locus (*ndv*B) of *R. meliloti* and the genomic DNA of *B. japonicum* (Dylan et al. 1986; Bhagwat et al. 1992), thus no DNA probe was available for identification and cloning of the gene(s) in *B. japonicum.* Considering a possible common role for β-glucans in plant-microbe interactions, we hypothesized that β-(1→3),β-(1→6)-D-glucan synthesis genes of *B. japonicum* could be identified by mobilizing a gene library to an *ndv*B mutant of *R. meliloti* and screening the exconjugants for functional complementation such as growth and motility on low osmolarity media followed by screening for their ability to form nitrogen-fixing

nodules on the legume host. These experiments demonstrated that the putative β-(1→3),β-(1→6)-D-glucan synthesis locus from *B. japonicum* on cosmid clone p5D3 is capable of functionally complementing an *ndv*B mutant of *R. meliloti* (Bhagwat et al. 1993).

In this paper, as one step in the elucidation of the synthesis and role of β-glucans, we used gene-directed Tn*5* mutagenesis to delimit the region encoding the synthesis of β-(1→3),β-(1→6)-D-glucans on cosmid clone p5D3, created a *B. japonicum* mutant deficient in the synthesis of β-glucans, and determined the plant phenotype induced by the mutant.

RESULTS

Identification of β-(1→3),β-(1→6)-D-glucan synthesis locus on p5D3.

To delimit the β-(1→3),β-(1→6)-D-glucan synthesis locus, several subclones of p5D3 were mobilized to the *ndv*B mutant of *R. meliloti*, TY7 (Dylan et al. 1986). The ability of the exconjugants to synthesize glucans was examined using in vivo and in vitro assays. Plasmid p115 (Fig. 1), which has 7.9-kbp internal *Hin*dIII fragments deleted, was the only subclone which synthesized β-(1→3),β-(1→6)-D-glucans and was selected for site-directed Tn*5* mutagenesis (Ditta 1986). Cosmids p115 carrying Tn*5* insertions at various locations (Fig. 1) were individually mobilized to *R. meliloti* TY7 and the exconjugants were screened for their ability to synthesize glucans in vitro. Membrane preparations from exconjugant strains of TY7 (p115::Tn*5*) were isolated and tested for their ability to incorporate UDP-(^{14}C)-Glucose into the soluble neutral fraction (Bhagwat et al. 1993). As illustrated in Table 1, TY7 exconjugants carrying Tn*5* insertions 32, 14, and 25 on p115, synthesized little or no glucan, and the adjacent insertions, 33 and 40, synthesized a reduced amount compared to the other Tn*5* insertions. The motility of the exconjugants was assayed using broth medium of low osmolarity in glass tubing as described by Kreig and Gerhardt (1994). The exconjugants carrying Tn*5* insertions 33, 32, 14, 25, and 40 on p115 were defective in their motility and were symbiotically ineffective on alfalfa (*Medicago sativa* cv. Sarnac). Therefore, the β-(1→3),β-(1→6)-D-glucan synthesis locus is most probably on the 5.2-kbp *Eco*R1 fragment of p115.

Isolation and characterization of an *ndv*B mutant.

The 5.2-kbp *Eco*R1 fragment from p115 carrying Tn*5* insertion 14 was subcloned in pSUP202 and mobilized to *B. japonicum* USDA 110 by triparental mating. Recombinants were obtained on AG medium containing 100 mM fructose (osmolyte) and the antibiotics kanamycin and streptomycin (200 μg/ml each). When recombinants were selected without fructose, most of the colonies were resistant to tetracycline, indicating integration of the vector pSUP202 into the genome. Genomic DNA was isolated from the colonies selected for tetracycline sensitivity and which did not hybridize with pSUP202 DNA (see Materials and Methods). The DNA was digested with *Eco*RI and Southern blot analysis performed (data not shown).

Using the 5.2-kbp *Eco*RI fragment from p115 as a probe, hybridization with a band at 11-kbp was observed in the putative mutant, AB-14. Using a 3.3-kbp internal *Hin*dIII fragment from Tn*5* as a probe, hybridization was observed with the 11-kbp band in strain AB-14, indicating that the 5.2-kbp *Eco*RI fragment carrying the putative *ndv*B locus was replaced with the corresponding DNA region containing Tn*5* insertion 14 (Fig. 1).

Characterization of *putative ndv*B mutant.

The mutant had a longer lag period in liquid AG medium before entering the logarithmic growth phase as compared to

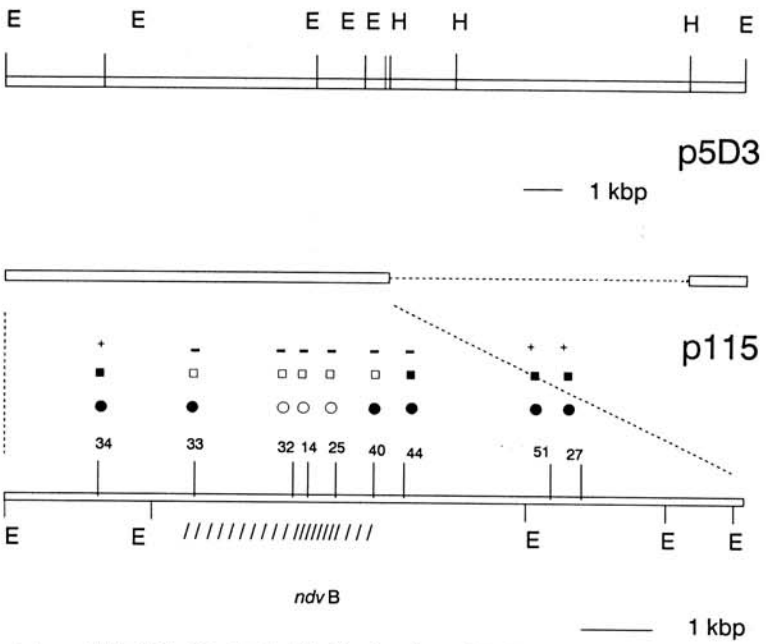

Fig. 1. Cosmid clone p5D3 and subclone p115. E, *Eco*RI; H, *Hin*dIII. The locations of Tn*5* insertions as a result of site-directed mutagenesis are indicated. Individual Tn*5* insertions on p115 were mobilized to *Rhizobium meliloti* TY7 to examine in vitro glucan synthesis. The phenotype of each Tn*5* insertion is shown. Synthesis (●) or absence (O) of in vitro glucan synthesis. Motile (■) or nonmotile (□) in low osmotic media. Symbiotically effective (+), or ineffective (–). The shaded area indicates possible location of the *ndv*B locus.

the wild type. This growth phenotype was corrected by addition of 100 mM fructose to AG medium (data not shown). Culture densities during stationary phase were similar. The mutant essentially was nonmotile in either the presence or absence of 100 mM fructose (Fig. 2).

β-Glucan synthesis in strain AB-14 was examined in vivo and in vitro. As illustrated in Table 2, no in vivo glucan synthesis was detected in the mutant strain and in vitro glucan synthesis was reduced more than 90% compared with the wild-type strain.

Strain AB-14 formed ineffective (small and white) nodules on soybean and host plants were visibly nitrogen deficient (yellow and stunted) when examined 30 days after inoculation (data not shown). The nodules were visibly deficient in leghemoglobin.

DISCUSSION

The ability of cosmid clone p5D3, which we previously isolated from a *B. japonicum* gene library, to functionally complement *R. meliloti* TY7, an *ndv*B mutant, which does not synthesize β-(1→2)-glucans, was used as an assay in delimiting the DNA region coding for β-glucan synthesis on p5D3. Site-directed Tn*5* mutagenesis was used to identify a 6.0-kbp region on p115 responsible for complementation of TY7 (Fig. 1, Table 1).

Table 1. In vitro glucan synthesis by *Rhizobium meliloti* TY7 exconjugants

Strain	In vitro glucan synthesis[a]
R. meliloti 102F34	2.67
TY7	0.01
TY7(p5D3)	1.85
TY7(p115)	2.72
TY7(p115::Tn*5*#34)	2.46
TY7(p115::Tn*5*#33)	1.60
TY7(p115::Tn*5*#32)	0.01
TY7(p115::Tn*5*#14)	0.01
TY7(p115::Tn*5*#25)	0.01
TY7(p115::Tn*5*#40)	1.26
TY7(p115::Tn*5*#44)	2.35
TY7(p115::Tn*5*#51)	2.67
TY7(p115::Tn*5*#27)	3.28

[a] Average of two experiments; nmoles of glucose incorporated/h/mg protein.

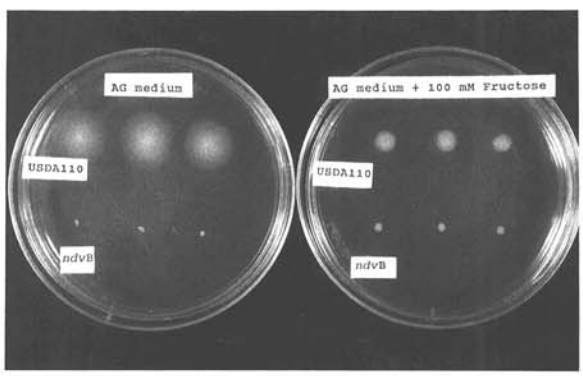

Fig. 2. Motility of a *Bradyrhizobium japonicum ndv*B mutant on semisolid medium. Cultures of *B. japonicum* wild-type strain USDA 110 and *ndv*B mutant AB-14 were spot inoculated on semisolid (0.35% agar) AG medium with or without 100 mM fructose. The plates were photographed 3 days after inoculation.

Several attempts to generate homologous recombination at the *ndv*B locus in *B. japonicum* by selection on AG medium with kanamycin and streptomycin, but without added osmolyte, were unsuccessful. Most likely this was due to the requirement of β-(1→3),β-(1→6)-D-glucan synthesis for normal growth in standard growth medium. Vector integration was observed in most of the 5,000 recombinants selected on AG medium. However, when fructose was included in the medium as an osmoprotectant, several colonies resulting from a homologous recombination were obtained. This is consistent with the suggested requirement for β-glucans as periplasmic osmoprotectants during growth under low osmotic conditions (Miller et al. 1986; Tully et al. 1990; Miller and Gore 1992; Pfeffer et al. 1994).

Southern blot analysis clearly demonstrated successful site-directed mutagenesis at the *ndv*B locus and this was confirmed by the inability of the mutant strain AB-14 to synthesize β-glucans in vivo or in vitro (Table 2). Strain AB-14 also was defective in its symbiotic interaction with soybean roots, forming small ineffective nodules. Kanamycin- and streptomycin-resistant bacteria were isolated from the nodules, and upon reinoculation, again formed only ineffective nodules. This is in contrast to nodules formed by *ndv*B mutants of *R. meliloti* and *R. fredii* from which no bacteroids could be isolated from the knotlike nodule structures (Dylan et al. 1986; Bhagwat et al. 1992).

In further characterizing the β-glucan synthesis genes, we plan to study the interruption of nodule development by *ndv*B mutants of *B. japonicum* and compare them with the *ndv*B mutants of *R. meliloti*. We plan to sequence the *ndv*B locus and to identify the *ndv*A-like locus which appears to be on p5D3.

MATERIALS AND METHODS

Organisms, growth, and nodulation assays.

Bradyrhizobium japonicum USDA 110 was obtained from the USDA-ARS National *Rhizobium* Culture Collection, Beltsville, MD. *R. meliloti* 102F34 and TY7 were obtained from S. Stanfield (Ielpi et al. 1990). *B. japonicum* cells were grown on minimal medium containing arabinose plus gluconate (AG medium, Cole et al. 1973), *R. meliloti* was grown on TY medium and *Escherichia coli* on LB medium (Sambrook et al. 1989). All media were supplemented with antibiotics when appropriate.

The growth rate was monitored turbidimetrically at A_{410}. Motility was determined by spot inoculation of AG medium containing 0.35% Difco agar and observing growth after 3 days at 28°C. Motility of exconjugants was confirmed using the method of Krieg and Gerhardt (1994) in which migration

Table 2. Glucan synthesis by putative *ndvB* mutant of *Bradyrhizobium japonicum*

Strain	In vivo glucan[a]	In vitro glucan synthesis[b]
USDA 110	100	5.85
AB-14	0	0.05

[a] μg hexose/mg protein. Cells were grown for 3.5 days and harvested during late log phase.
[b] nmoles of glucose incorporated/h/mg protein.

is observed after inoculation into a glass tube containing semisolid medium.

Soybean (*Glycine max* cv. Williams) and alfalfa (*Medicago sativa* cv. Sarnac) plants were grown in modified Leonard jars assemblies and after inoculation were maintained for 4 weeks on nitrogen-free Jensen's nutrient solution (Vincent 1970) in a greenhouse. Nodules were examined 3 to 4 weeks after inoculation.

Assays for glucan synthesis.

B. japonicum cells were grown in AG medium, harvested during late log phase, and the glucans were extracted with 1% TCA and purified as previously described (Bhagwat et al. 1992). The neutral glucan fraction obtained after DEAE-cellulose chromatography was purified further using chromatography on a C_{18} silica reverse-phase column as described by Rolin et al. (1992). The purity of the glucans obtained using this procedure was comparable to those obtained by these authors (P. E. Pfeffer, personal communication).

For in vitro glucan synthesis, total cytoplasmic membranes were prepared and assayed using UDP-[^{14}C(U)]glucose (266 mCi/mmol) as described by Bhagwat and Keister (1992).

DNA techniques.

Site-directed Tn*5* mutagenesis of cosmid p115 was carried out as described by Ditta (1986). The location of individual Tn*5* insertions was determined by restriction endonuclease analysis. Insertions were mobilized to *R. meliloti* TY7 by triparental mating (Ditta 1986). For homologous recombination at the *ndv*B locus, a 5.2-kbp *Eco*RI fragment carrying the Tn*5* insertion (#14) was subcloned into pSUP202 and mobilized to *B. japonicum* USDA 110 by triparental mating. Colonies were screened for loss of pSUP202 essentially as described by Fu and Maier (1993). For screening several hundred colonies, the putative recombinant *B. japonicum* USDA 110 colonies were picked from AG agar medium containing antibiotics (kanamycin and streptomycin, 200 µg/ml each) and 100 mM fructose, and grown in 96-well microtiter plates containing 100 µl of AG broth with antibiotics (kanamycin and streptomycin, 100 µg/ml each) and 100 mM fructose. Cultures from these wells were examined for loss of tetracycline resistance by stamping (with a 96-prong probe) onto AG medium with 100 mM fructose and tetracycline at 100 µg/ml. Colonies which failed to grow were selected from the original well in the microtiter plate. The exconjugant colonies were further purified on AG medium containing kanamycin and streptomycin (200 µg/ml each), and the resulting colonies were further screened for the loss of vector (pSUP202) by performing colony hybridization with ^{32}P-labeled pSUP202 DNA (Sambrook et al. 1989; Fu and Maier 1993). Total genomic DNA was isolated from colonies which were tetracycline sensitive (and resistant to kanamycin and streptomycin), and which did not hybridize with pSUP202 (Sadowsky et al. 1987). The position of Tn*5* in the chromosome was determined by Southern blot analysis. Restriction endonucleases were purchased from United States Biochemical Corp. and New England Biolabs and were used according to the specifications of the manufacturer. Restriction fragments were separated by horizontal electrophoresis on 0.7% agarose gels in Tris-acetate buffer (Sambrook et al. 1989). For hybridizations, DNA was transferred to Nytran membranes (Schleicher & Schuell) and probed using ^{32}P-labeled DNA made by random primer labeling (Sambrook et al. 1989).

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