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Research Note

Isolation and Characterization of a Locus from *Azospirillum brasilense* Sp7 That Complements the Tumorigenic Defect of *Agrobacterium tumefaciens chvB* Mutant

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The chromosomal virulence gene *chvB* of *Agrobacterium tumefaciens* is required for pathogenesis. A DNA fragment from the *chvB* locus can hybridize to DNA from *Azospirillum brasilense* Sp7. This DNA fragment could restore the tumorigenic activity of the *chvB* mutant strain *A. tumefaciens* A1011 towards leaf disks of *Nicotiana tabacum*. An NH_2-terminal open reading frame, 480 codons long, was most likely responsible for the restoration of the tumorigenic activity. The *A. brasilense* sequence showed good homology with the NH_2-terminal region of the *ndvB* gene of *Rhizobium meliloti*.

Additional keywords: DNA base sequence, *lacZ* fusion, *Rhizobium meliloti ndvB* locus.

The diazotroph *Azospirillum brasilense* can adsorb to root hairs of moncotyledonous plants (Elmerich 1984; Kapulnik et al. 1985; Okon 1985) and also to individual cells (Eyers et al. 1988). *Agrobacterium tumefaciens* induces tumors on dicotyledonous plants, while *Rhizobium meliloti* induces nitrogen-fixing nodules on the roots of alfalfa plants. The processes of attachment and infection by both bacteria require, in addition to genes encoded by resident plasmids, a related set of chromosomal genes designated *chvA* and *chvB* in *A. tumefaciens* (Douglas et al. 1985) and *ndvA* and *ndvB* in *R. meliloti* (Dylan et al. 1986). DNA fragments containing the *ndvA* and *ndvB* genes can complement mutations in *chvA* and *chvB*, respectively, in *A. tumefaciens* (Dylan et al. 1986).

Could there be a genetic locus in *A. brasilense* that is analogous to *chvB* and *ndvB*? From the results of Southern blot hybridization of genomic DNA of several *Azospirillum* species including Sp7, Altabe et al. (1990) reported that these species did not have *chvB* homologous sequences. Contrary to this, Waelkens et al. (1987) had reported that genomic DNA from *A. brasilense* Sp7 hybridized with probes containing the *chvB* locus of *A. tumefaciens*.

We report here that *A. brasilense* Sp7 does have a locus homologous to the *chvB* locus of *A. tumefaciens*, and the cloned *A. brasilense* locus can complement a *chvB* mutational defect in *A. tumefaciens* with respect to tumor formation in leaf disks of *Nicotiana tabacum*. The segment of DNA involved in complementation can encode only a 480 amino acid-long NH_2-terminal polypeptide fragment that has sequence homology with the NH_2-terminal end of NdvB protein of *R. meliloti*.

A sequence homologous to a 1.25-kb *Eco*RI fragment internal to the *chvB* locus (Douglas et al. 1985) was found in the *A. brasilense* Sp7 genome by Southern hybridization. These were a 6.5-kb *Eco*RI fragment, a 5.1-kb *Bam*HI fragment, and a 4.6-kb *Hin*dIII fragment (data not shown). Restriction analysis revealed that these three were overlapping fragments (Fig. 1).

Complementation studies were done to determine if the *chvB*-homologous DNA from *A. brasilense* constituted a locus functionally equivalent to the *chvB* locus. The recombinant plasmids pSR12 (6.5-kb *Eco*RI fragment cloned in pRK405) and pSR14 (9.5-kb *Eco*RI–*Hin*dIII fragment cloned in pRK405) were mobilized into *A. tumefaciens* A1011 (*chvB*::Tn*5*) from *E. coli* S17.1 (Simon et al. 1986) by conjugation. The vector pRK405 is a derivative of pRK404 (Ditta et al. 1985) in which the *Eco*RI site outside the polylinker has been destroyed by filling. The exconjugants were used to infect the *Nicotiana tabacum* leaf disks. The *A. tumefaciens* strains A348 (wild type) and A1011 (*chvB*::Tn*5*) (Douglas et al. 1985) were used as positive and negative controls, respectively. The defect due to *chvB* mutation in the strain A1011 could be complemented by both pSR12 and pSR14 plasmids, since virulence was exhibited towards tobacco leaves (Fig. 2). We propose to designate the *A. brasilense* locus *cviB* (*c*omplements *vi*rulence). The sequence on the right side of the broken arrow in the restriction map (Fig. 1) may not be essential for virulence, since pSR12 can also complement the defects of the *chvB* mutant.

The DNA sequence of both strands of the segment between the solid vertical arrows (Fig. 1) was determined by the dideoxy chain termination method (Sanger et al. 1977). An open reading frame (ORF) was found, which was preceded by the two terminator codons TAA and TAG (Fig. 3, underlined). The first ATG in this frame (underlined) has been assumed to be the translation initiatior codon. An imperfect Shine and

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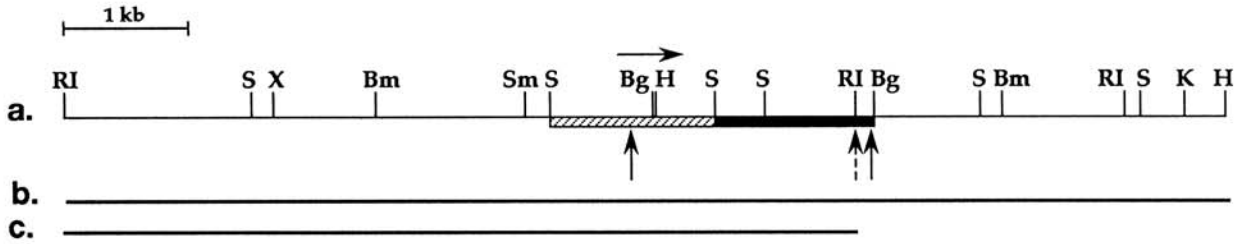

Fig. 1. A, Restriction endonuclease map of the 9.5-kb DNA containing *cviB*. The hatched and the black rectangles represent the regions homologous to the 1.25-kb *Eco*RI fragment internal to the *chvB* locus of *Agrobacterium tumefaciens*. The *Sal*I fragment (hatched rectangle) was used for constructing the *cviB*::*lacZ* promoter fusion. The horizontal arrow represents the direction of transcription. DNA sequence has been determined for the region between the two solid vertical arrows. The region on the right hand side of the broken arrow is not necessry for the tumorigenic activity. **B,** The 9.5-kb *Eco*RI/*Hin*dIII fragment contained in pSR14. **C,** The 6.5-kb *Eco*RI fragment contained in pSR12. Abbreviations: RI, *Eco*RI; S, *Sal*I; X, *Xho*I; Bm, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; Sm, *Sma*I.

Dalgarno sequence (CGAGC, broken underlined) exists immediately upstream. There is a GTG (underlined) in the same frame, upstream of the putative initiator codon ATG, but there is no semblance of any Shine and Dalgarno sequence preceding it. The ORF extends beyond the sequenced region that ends in a *Bgl*II site (Fig. 3, boxed). The NH_2-terminal segment of the putative CviB polypeptide from *A. brasilense* capable of correcting the *chvB* mutant phenotype with respect to virulence, is only 480 amino acide long.

The direction of transcription was confirmed by constructing a *cviB*::*lacZ* promoter fusion. A 1.4-kb *Sal*I fragment (hatched rectangle, Fig. 1) was cloned into the broad host range transcription fusion vector pGD499 (Ditta et al. 1985) in both orientations. The construct with the promoter in the correct orientation, in which the *Hin*dIII site within the *Sal*I fragment was closer to the *lacZ* gene (pSR22.2) and the construct with the promoters in opposite orientation (pSR21.2), were mobilized into *A. brasilense* Sp7 by conjugation. The colonies of exconjugants with pSR22.2 and pSR21.2 elicited β-galactosidase activities of 2,120 and in 180 Miller units (Miller 1972), respectively. Thus, the promoter of *cviB* is likely to be within the 1.4-kb *Sal*I fragment, and the direction of transcription is from left to right (horizontal arrow, Fig. 1).

The DNA sequence of the *cviB* locus was found to have considerable homology with the relevant portion of the *ndvB* gene of *R. meliloti* (Ielpi et al. 1990), the gene that could also complement the defect in the *chvB* locus of *A. tumefaciens*. The homology was poor, with bases upstream of the base number 32 (underlined C, Fig. 3), which corresponded to base number 377 designated by Ielpi et al. (1990) in the *R. meliloti ndvB* sequence.

The derived amino acid sequence of CviB had 60% homology with the derived amino acid sequence of NdvB at the level of identical amino acids and 76% homology if similar amino acids were considered. However, the first 38 amino acids at the NH_2-terminal end of NdvB were absent at the NH_2-terminal end of CviB (Fig. 4). The base sequence of the corresponding region has been rechecked by using the oligonucloetide primer, 5′-ATAGGACGCGCGGATCGAATC3′ (bases complementary to base numbers 87–67, Fig. 3). The hydropathy plot of the NH_2-terminal region of the putative CviB polypeptide, determined according to Kyte and Doolittle (1982), has revealed the presence of a possible transmembrane segment that corresponds to one of the transmembrane segments of the NdvB polypeptide (box, Fig. 4).

Since *A. brasilense* is not known to produce tumors or nodules in plants, but adsorbs to the roots, we speculate one of the functions of this locus is to participate in the adsorption.

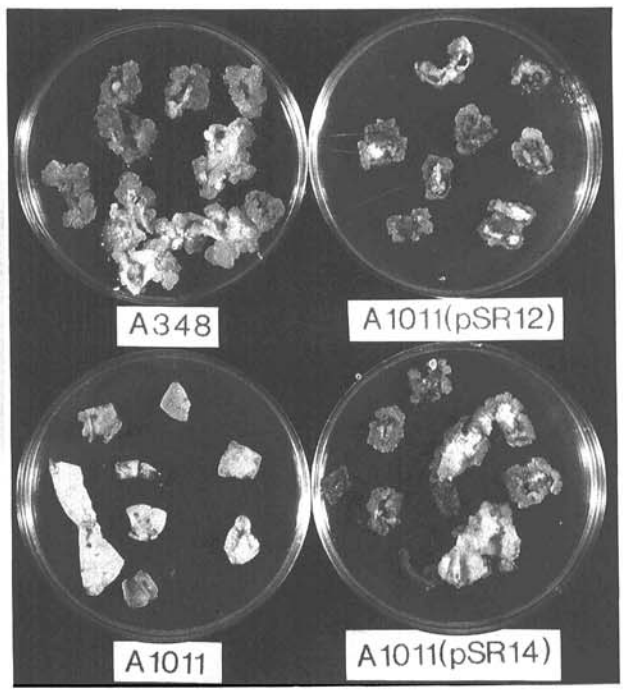

Fig. 2. Leaf disks of *Nicotiana tabacum* infected with *Agrobacterium tumefaciens* A348, wild type; *A. tumefaciens* A1011, *chvB*::Tn5; *A. tumefaciens* A1011/pSR12; and *A. tumefaciens* A1011/pSR14. The photograph was taken after incubation for a period of 20 days at 25° C. The leaf pieces were incubated on Murashige and Skoog (MS) basal medium (Himedia Laboratories, India) in a petri plate at 25°C in the dark for 24 hr. Cultures of different strains of *A. tumefaciens* grown overnight were centrifuged, and the cells were washed and resuspended in sterile water. The explants (six to eight for each strain) were submerged in the bacterial suspension for 10 to 15 min, soak dried on sterile blotting paper, placed back on the MS basal medium, and incubated at 25°C in the dark for 48 h. The explants were then removed from the plate and washed with autoclaved water, soak dried on a blotting paper, and placed back on MS basal medium containing the necessary antibiotics. Explants infected with the strains A348, A1011, A1011/pSR12, and A1011/pSR14 were placed on MS basal medium containing Cb (carbenicillin, 100 μg/ml); Cb and Km (kanamycin, 100 μg/ml); and Cb, Km, and Tet (tetracycline, 10 μg/ml); and Cb, Km, and Tet, respectively. The plates were then incubated in diffused light at 25°C for 20 days. After 10 to 12 days swellings from the edges were seen which grew into crown gall tumors.

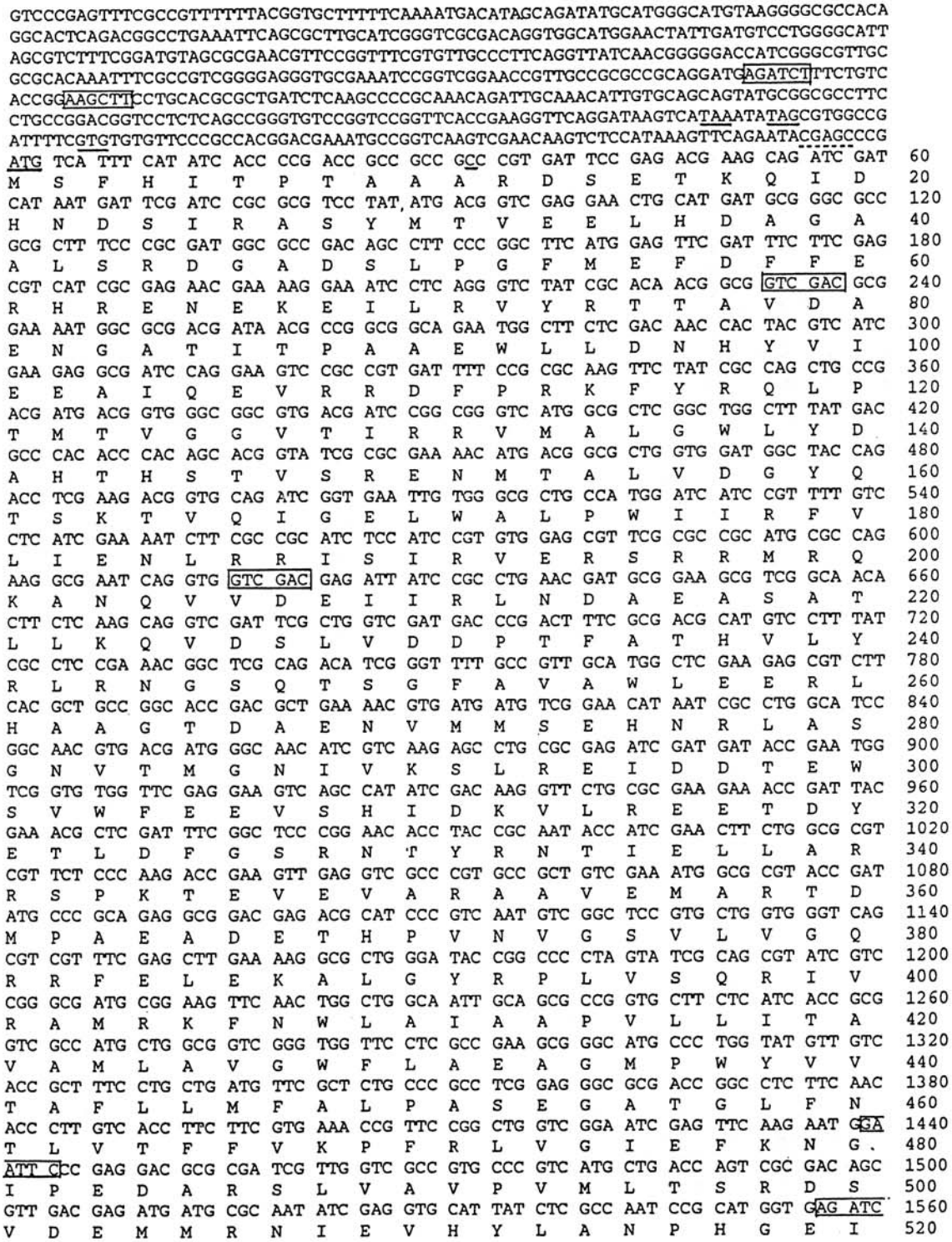

Fig. 3. Partial nucleotide sequence of the *Azospirillum brasilense cviB* locus. The deduced amino acid sequence is shown below the nucleotide sequence. The presumed initiator ATG is underlined as also the termination codons TAA and TAG upstream of initiator ATG. The imperfect Shine and Delgarno sequence is represented by the broken line under the sequence. The restriction endonuclease sites, *Bgl*II (AGATCT), *Hin*dIII (AAGCTT), *Sal*I (GTCGAC), and *Eco*RI (GAATTC) are boxed. Significant homology with the *ndvB* gene of *R. meliloti* begins at the base C at position 32 (underlined).

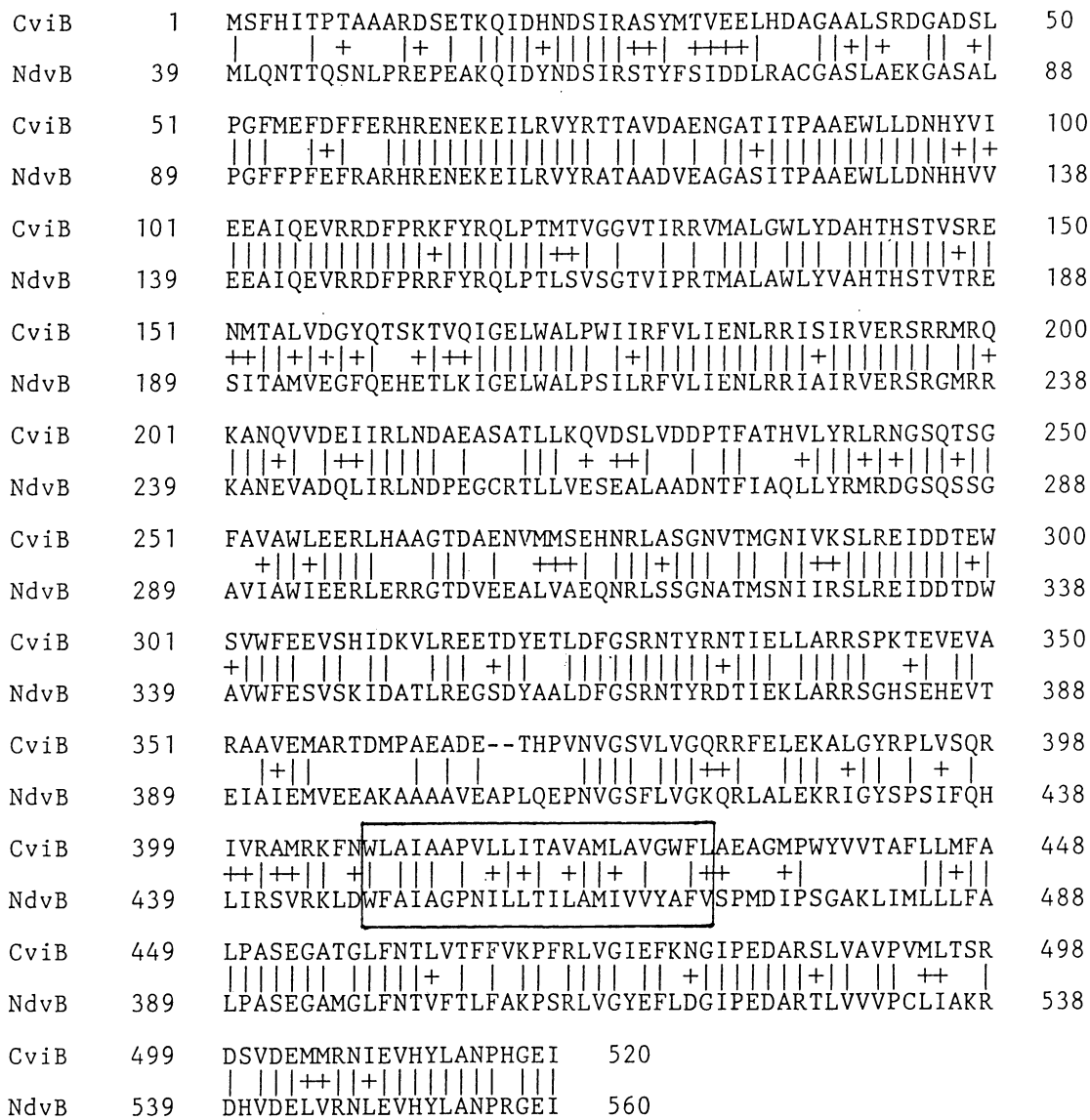

Fig. 4. Amino acid sequence comparison of NH_2-terminal regions of CviB and NdvB proteins. Identical amino acids are represented by vertical bars and the similar amino acids are represented by plus signs. The potential transmembrane domains deduced from the hydropathy plots of the two polypeptides are boxed.

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