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The Chromosomal Virulence Gene, *chvE*, of *Agrobacterium tumefaciens* Is Regulated by a LysR Family Member

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Certain plant phenolic compounds and monosaccharides induce the transcription of virulence (*vir*) genes of *Agrobacterium tumefaciens* through the VirA-VirG two-component regulatory system. The product of the chromosomal virulence gene *chvE* is homologous to galactose-binding protein of *Escherichia coli* and is required for *vir* gene induction by sugars. Adjacent to, but divergent in transcription from, *chvE* is an open reading frame, now termed *gbpR* (galactose-binding protein regulator), that is homologous to the LysR family of transcriptional regulators. *chvE*::*lacZ* expression was induced by L-arabinose, D-galactose, and D-fucose when *gbpR* was present. In the absence of inducer, GbpR repressed *chvE*::*lacZ* expression. In addition, GbpR negatively regulated its own expression.

Agrobacterium tumefaciens transforms plant cells and induces tumors on many dicotyledonous and some monocotyledonous plants by transferring a piece of DNA (T-DNA), a part of a large tumor-inducing (Ti) plasmid, into plant cells, where it becomes integrated and expressed in the plant cell genome. Specific phenolic compounds and monosaccharides generated from plant wound sites act synergistically to induce a set of bacterial virulence (*vir*) genes which are required for the processing as well as the transfer of T-DNA (reviewed in references 35 and 37). The expression of these genes is induced via a cascade of phosphorylation reactions involving a transmembrane sensor protein, VirA, and a transcriptional activator, VirG (21, 36). Both genes are members of a two-component regulatory system which bacteria commonly use to sense and react to environmental changes. (reviewed in references 6, 30, and 35).

In addition to the virulence genes on the Ti plasmid, several genes on the chromosome, some designated *chv*, are involved in the plant-pathogen interaction. One important chromosomal virulence gene whose function is largely understood is *chvE*. This gene codes for a periplasmic sugar-binding protein which is essential for *vir* gene induction by monosaccharides (3, 20). Induction by high levels of phenolics such as acetosyringone is unaffected in the A348 *chvE* mutant strain. However, the synergistic effect of monosaccharides on *vir* gene induction by low levels of phenolic compounds was not observed in *chvE* mutant strains. *chvE* mutants are avirulent on a variety of plants but can cause delayed tumor formation on others. It has been proposed that nonsusceptible host plants may lack a sufficient level of specific phenolic compounds to induce the *vir* genes. Therefore, tumor formation on these plants may require the synergistic effect of sugars on *vir* gene induction, which is only possible when *chvE* is expressed (3). In addition to its role in *vir* gene induction, *chvE* is necessary for normal growth on and chemotaxis towards certain sugars. Thus, ChvE plays an important role both in virulence as well as in the physiology of *Agrobacterium* spp. in general.

The nucleotide sequence of *chvE* is similar to that of the galactose-binding protein (GBP) of *Escherichia coli*, and the derived protein sequence is virtually identical to the corresponding amino-terminal protein sequence of GBP1 (galactose-binding protein) of *Agrobacterium radiobacter* (3, 8, 9, 20). In *E. coli*, GBP has been shown to interact directly with the membrane chemoreceptor protein Trg to trigger chemotaxis (24). By analogy, it seems likely that the *chvE* gene product interacts directly with a site identified on the periplasmic region of VirA that is similar to Trg to facilitate *vir* gene induction by sugars (3). Deletion of the entire VirA periplasmic domain (3) or deletion of this Trg-related region (unpublished data) results in a loss of *vir* gene induction by monosaccharides and a reduced host range similar to that of *chvE* mutants.

In the course of determining the DNA sequence of the *chvE* region, a portion of a second open reading frame (ORF1), divergent in transcription, was revealed (20). This sequence shows strong similarity to those of the LysR family of transcriptional regulators (19). All LysR family members share certain features; they contain a helix-turn-helix motif in the amino terminus which binds to DNA, they are usually transcribed divergently from the gene(s) they regulate, and several have been shown to be negatively autoregulated. Several members of the LysR family have been identified in members of the family *Rhizobiaceae*. For example, OccR, found on the Ti plasmid of octopine-type plasmids of *A. tumefaciens*, was shown to regulate genes required for the breakdown of the metabolite octopine, which is synthesized by crown gall tumors (18, 34). Another notable family member is the regulatory protein NodD of *Rhizobium* spp., which interacts with plant flavonoids to activate the *nod* genes, which are required for the formation of nodules on plant roots (17). On the basis of the sequence similarity of ORF1 to the LysR family and its proximity to *chvE*, it was hypothesized that ORF1 may regulate the expression of *chvE* (20). In this report, we confirm that ORF1, now termed *gbpR* (GBP regulator), not only regulates the expression of *chvE* but also regulates its own synthesis.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in Luria-Bertani broth supplemented when appropriate with the following (in micrograms per milliliter): ampicillin, 100; kanamycin, 100; gentamicin, 5; spectinomycin, 50; or tetracycline,

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Agrobacterium strains		
A348	C58 chromosome, octopine-type pTiA6NC	15
MX503	A348, *chvE*::Tn*3*-HoHo1 (*chvE*::*lacZ* fusion)	20
MX550	A348, *gbpR*::Tn*3*-HoHo1 (*gbpR*::*lacZ* fusion)	20
At12001	A348, *gbpR*^*nptI* (Km^r)	This study
At12002	A348, deletion at N-term; ^*nptII* (Km^r)	This study
UIA143	*autC58 recA*::Ery140 (pAtC58)	11
E. coli strains		
MT607	MM294A; *recA56*	13
MT616	MT607(pRK600); tra^+ mob^+ (Cm^r)	13
HB101(pRK2073)	tra^+ mob^+ (Sp^r)	10
HB101(pHoHo1, pSshe)	Tn*3lacZ*, *tnpA* (Cb^r, Cm^r)	29
Plasmids		
pGC31	pTZ19R, *gbpR* and *chvE* (Cb^r)	20
pGC31K	pTZ19R, *gbpR*^*nptI* and *chvE* (Cb^r, Km^r)	2a
pMWH503	pVK102, *chvE*::Tn*3*-HoHo1 (Cb^r)	20
pMWH550	pVK102, *gbpR*::Tn*3*-HoHo1 (Cb^r)	20
pTC110	IncW, derivative of pUCD2 (Km^r, Gm^r)	5
pTC116	pTC110, $gbpR^+$ $chvE^+$ (Km^r, Gm^r)	5
pTZ19R	*lacZ'*, f1 ori (Cb^r)	U.S. Biochemical Corp.
pUC19mob	pUC19, mob^+ (Cb^r)	27
pUCD1002	Broad-host-range cloning vector (Km^r)	14
pUFR047	IncW, mob^+ (Cb^r, Gm^r)	12
pVK101	IncP, mob^+ (Km^r, Tc^r)	22
pVK102	IncP, mob^+ cos^+ (Km^r, Tc^r)	22
pSL1	pUC19mob, *gbpR*^*nptI* (Cb^r, Km^r)	This study
pSL2L	pGC31, *gbpR* deletion at N-term;^*nptII*	This study
pSL3	pUC19mob, *Eco*RI fragment from pSL2L (Km^r, Cb^r)	This study
pSL7	pUCD1002, *gbpR* region from pGC31 (Km^r)	This study
pSL8	pUFRO47, *gbpR* and *chvE* from pGC31 (Cb^r, Gm^r)	This study
pSL10	pTZ19R, *Pst*I-*Sma*I of *gbpR* (Cb^r)	This study
pSL20	pGC31 with deletion of *gbpR* (Cb^r)	This study
pSL25	pVK101, *chvE* region from pSL20 (Km^r)	This study
pSL36	pGC31 with *gbpR-chvE* insert reversed	This study
pSL43	pUFRO47, *gbpR* deletion and *chvE* from pSL2L (Km^r, Cb^r, Gm^r)	This study

[a] Abbreviations: Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Er^r, erythromycin resistance; Gm^r, gentamicin resistance; Cm^r, chloramphenicol resistance; Sp^r, spectinomycin resistance; ^, insertion; N-term, amino terminus.

15. *Agrobacterium* strains were grown at 28°C in MG/L medium (7) or ABG medium, which is AB minimal medium (4) modified with 0.5% glycerol substituting for glucose. Antibiotics were used at the following concentrations (micrograms per milliliter): carbenicillin, 100; kanamycin, 100; tetracycline, 5; gentamicin, 100; or erythromycin, 150. All chemicals used were obtained from Sigma Corp., except acetosyringone (Aldrich), MES (morpholineethanesulfonic acid [Research Organics]), D-(+)-glucose (EM Science), glycerol (J. T. Baker, Inc.), and gentamicin and carbenicillin (U.S. Biochemical Corp.). Enzymes were purchased from GIBCO BRL, except for T4 polynucleotide kinase (U.S. Biochemical Corp.).

DNA sequencing. The 1.2-kb *Sma*I-*Pst*I fragment containing the 3′ region of *gbpR* was subcloned from pGC31 into pTZ19R, creating pSL10. The forward strand was sequenced with Reverse Primer and one internal primer and the Sequenase version 2.0 kit from U.S. Biochemical Corp., which were used according to the instructions provided by the manufacturer for single-stranded DNA sequencing. The reverse strand was sequenced with the double-stranded sequencing method, and an oligonucleotide was designed with sequence information from the forward strand.

Primer extension analysis of *gbpR* and *chvE* transcripts. A348(pSL8) was grown on AB plates with gentamicin. After 3 days, 2 ml of ABG medium with gentamicin was inoculated and the cultures were grown overnight. A total of 1.6 ml of culture was used to inoculate 40 ml of fresh medium. After the cultures reached a cell density of 0.6, as determined by A_{600}, the cells were pelleted and resuspended in 20 ml of ABG with carbenicillin with or without 10 mM L-arabinose. After 2 h of incubation at 28°C with shaking, total RNA was extracted (31) and primer extension analysis was performed as described previously (32). Fifteen micrograms of RNA from cells grown in each culture condition was hybridized with ^{32}P-labeled oligonucleotides (30-mers) complementary to either *gbpR* or *chvE* mRNA. The sequences of the oligonucleotides were as follows: 5′-GGACGCAGCACCGATGGCACAAGCTGC CAT-3′ for *chvE* and 5′-GCTGACCTGTCCGTGCTCCTCG ATCATCAC-3′ for *gbpR*. Following incubation with Moloney murine leukemia virus reverse transcriptase, labeled transcripts were size fractionated on a 6% polyacrylamide gel, which was dried and visualized by Phosphor Imaging (Molecular Dynamics).

Induction of *chvE*::*lacZ* and *gbpR*::*lacZ* gene fusions. Strains were grown for 2 days in 2 ml of MG/L medium supplemented with carbenicillin. The cells were pelleted, washed, and resuspended in 2 ml of ABG medium. Sixty microliters of this washed suspension was inoculated into 3 ml of ABG medium alone or with 5 or 10 mM sugars. Tubes were incubated for 24 h with shaking, and β-galactosidase activity was assayed as

described previously (25, 29). Units of β-galactosidase activity were calculated with the equation (1,000 × A_{420})/(time × A_{600}) as described previously (25). All experiments involving β-galactosidase assays were repeated at least three times in triplicate.

Mutagenesis of *gbpR*. The *gbpR* gene was disrupted by cloning a 1.5-kb kanamycin resistance cartridge (Pharmacia) into the *Nar*I site 282 bp downstream of the start codon of *gbpR*. The *Eco*RI fragment of this resultant plasmid (pGC31K), containing *chvE* and the disrupted *gbpR* gene, was then cloned into the *Eco*RI site of pUC19mob, which cannot replicate in *Agrobacterium* spp. This plasmid, pSL1, was then introduced into A348 by triparental mating (10) with HB101 (pRK2073). Kanamycin-resistant colonies were screened for gene replacement as judged by sensitivity to carbenicillin. Double-crossover events were verified by Southern hybridization (28). The resulting *gbpR* mutant was designated At12001.

A second mutant, At12002, was constructed by deletion of a portion of the *gbpR* gene. The *Sst*II site downstream of *gbpR* was eliminated by partial digestion of pGC31 with *Sst*II, creation of blunt ends with T4 DNA polymerase, and religation. The 225-bp *Nar*I-*Sst*II fragment containing the helix-turn-helix motif was then removed and replaced with the *Hin*dIII fragment of Tn*5* containing the *npt*II gene by blunt-end ligation. pSL2L contains the *npt*II gene oriented away from *chvE*. The *Eco*RI fragment was cloned into pUC19mob, and this plasmid (pSL3) was used as described above to create a deletion mutation in A348 (At12002).

Generation of a *chvE*::*lacZ* gene fusion on a plasmid lacking *gbpR*. The *gbpR* gene was deleted from pGC31 by digestion with *Sst*II and religation to generate pSL20. The 2.1-kb *Sal*I fragment of pSL20 containing *chvE* and its promoter was ligated into pVK101, creating pSL25. Transcriptional fusions between *chvE* and *lacZ* were generated by random Tn*3*-HoHo1 mutagenesis of pSL25 as described by Stachel et al. (29) with the following modifications. Triparental mating between HB101(pHoHo1, pSshe1, pSL25), the *Agrobacterium* strain UIA143, and HB101(pRK2073) was carried out, with selection for erythromycin, carbenicillin, and kanamycin resistance on medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Plasmids from blue colonies were then transferred to *E. coli* MT607 by triparental mating with MT616 as a mobilizer strain. A plasmid with a Tn*3*-HoHo1 insertion in *chvE* was identified by restriction mapping and was designated pSL25/$chvE_{101}$::Tn*3*-HoHo1. This plasmid was introduced into A348, At12001, and At12002 by triparental mating.

chvE expression was measured as follows. Strains were grown for 4 days on AB plates containing carbenicillin and kanamycin. Two milliliters of ABG medium with carbenicillin was inoculated in triplicate with each strain, and the cultures were grown for 2 days. Sixty microliters was then inoculated into 3 ml of ABG medium containing carbenicillin with and without 10 mM L-arabinose. Cultures were grown for 24 h, and β-galactosidase activity was measured as described previously (29).

***trans*-Complementation of the *gbpR* mutants.** pTC116 and the vector control pTC110 were introduced by electroporation (4) into A348, At12001, and At12002, each containing pSL25/$chvE_{101}$::Tn*3*-HoHo1. β-Galactosidase activity was measured as described above.

Plasmid construction for *gbpR* autoregulation study. The 1.9-kb *Kpn*I-*Pvu*II fragment containing *gbpR* from pGC31 was isolated, and pUCD1002 was cut with *Kpn*I. Blunt ends were created on each linear fragment with T4 RNA polymerase prior to ligation, resulting in pSL7.

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1    CATTAACTCT CTCCCTTGAG CCCTTCTCGG AGCCCGCATG CTGCGGTGCA
51   CCATGAAATT CATGCCGTCA CAAGTAAGGA GCCGCCCTGC GCTTGCTCCA
101  TTTGCAGTTG CGAAATGAAA CAGCTGCCGC TCCTCCCCGG CATTCCGCAA
151  ATTGTTCTGA ATCCATATAA CTGTCAAATA GAATATATCG GTTTCCATAT
201  ATCAATTATG ATATATTAAA TCGATACAAA AACCGATGAG GGGTATAAAA
251  TCAAGGATGA GCAACCAAAA AGTGTATGAG CAGGCGCAAA CAACAGAGGC
301  TCTTTTCGTC TTTGAAGACA ACCCTCTGCT GCGGGCCGGA TTAAAGATG
350  AGCCACCTGC GCATGCTCGT GATGATCGAG GAGCACGGAC AGGTCAGCGC
400  CGCCGCGGCA GCCATGAACA TGACGCAGCC GGCAGCCTCG CGCATGCTCT
450  CGGAAATGGA AGCAATCGTC AAATCACCGC TTTGTCAGAG AGCCTCGCGC
500  GGCGTGGTGC TGACGAAATT CGGCGAGGCG CTGGCGCTGG CGCGCCGCGC
550  CCGCACCATA TTGCTGGAAC TGCGCGAAGC TAGCCGTGAA CTCAACCAGA
600  TGAAGTCGGG CAGCGGCGGT TCGGTCTATA TCGGCGCCGT CACCGCCCCG
650  GCGATCAGCC TCGTCGTTCC CGCCATCAGG CGCGCCATGG ACACCTATCC
700  CGGCATCGAA ATCAACGTGC AGGTGGAGAG CAGCAACGTG CTGGCCCGGG
750  AACTGCTGGC GGCGCGTCAC GATTTCATCA TCGGCCGCAT TCCCGATGAC
800  TTCGATCCCG GCCTCTTCTC CATCCACGAA ATCGGCATCG AGCGCGCCTG
850  TCTGGTGGTG CGCGAAGGCC ACCCGCTGAT GCGCGGCGAG CCCGTGACCC
900  TGCAGGATCT CTCCGGTTAC GACTGGGTCT TCCAGCCACC CGGCGCGCTT
950  TTGCGCAGAA CGATGGAGGA TGTCTTCCTC ACCCATGGCG TCGCCATGCC
1000 GCGCAACGTC ATCAACACGC CATCCGTGGT GCTCACCCTT GCCCTCGTCT
1050 GCAATACCAA TGCCATCGCC CCCATCGCGC AGGACATGGC CGAATTTGTT
1100 GCGGGGCAGC AGGCAGATAT GGCCGAACCC GCATTTTGCC AACGGATTTC
1150 GAACTCGTGG TCAAGCCTTA CAGCATCATT ACCACCAAAG GCCGGGTCCT
1200 GCCGCCCAGC GCCCGCCTGC TTTATGATCT GGTGCTGGAT GAAAGCCGTA
1250 AGCTGACCAA CACCTGACGA CGGCAAGCCC GACTCCACCT TCACCCTTTA
1300 TAGAAGCCGG ATCATTGAGG CCGGCGC
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FIG. 1. Nucleotide sequence of *gbpR*. The putative start and stop codons for GbpR are in boldface. The start codon for *chvE* (TAC, negative strand) is underlined. The putative ribosome binding sequences are double underlined. The A+T-rich region is boxed. The transcriptional start site for *chvE* is marked with asterisks. The LysR family DNA binding motif T-N_{11}-A is underlined with a dashed line. Arrows indicate the direction of transcription.

Nucleotide sequence accession number. The nucleotide sequence of *gbpR* has been submitted to GenBank under accession no. L10424.

RESULTS

***gbpR* sequence analysis.** Huang et al. reported that an ORF (ORF1) was located next to, but divergent in orientation of transcription from, *chvE* (20). A partial sequence of ORF1 revealed that it had significant similarity to the LysR family of transcriptional regulators (19). In virtually all cases studied, members of this family are transcribed divergently from the genes they regulate, and on this basis Huang et al. proposed that ORF1 may regulate the expression of *chvE*. In order to further characterize ORF1, we completed the DNA sequence and later designated it *gbpR*. The putative start codon ATG is at position 347 (Fig. 1) and is preceded 6 bp upstream by a likely ribosome binding site (GGA). A helix-turn-helix motif characteristic of the LysR family is located in the amino-terminal region from amino acids 16 to 35 (20). On the basis of the start codon at position 347 and the stop codon at position 1254, the *gbpR* gene is 909 bp in length and would encode a peptide 303 amino acids in length.

A 29-nucleotide A+T-rich region was found in the 345-bp intergenic region between *chvE* and *gbpR* (Fig. 1). This se-

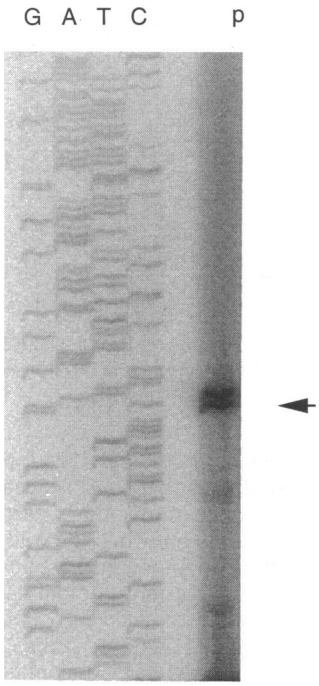

FIG. 2. Primer extension analysis to determine the transcription start site for *chvE*. An end-labeled oligonucleotide complementary to the *chvE* transcript was used to prime reverse transcription of RNA isolated from A348(pSL8). Products were separated by electrophoresis in a sequencing gel adjacent to sequencing ladders, labeled G, A, T, or C, generated with the same primer on single-stranded pSL36 DNA. The primer extension product in lane p is indicated with an arrow.

quence, AATTATGATATATTAAATCGATACAAAAA, is 86% A+T and is located 107 bp from the putative translational start site for *gbpR*. Similar A+T-rich regions have been found in the intergenic regions of other LysR family members (33). The function, if any, of these A+T-rich regions is not known, but it has been proposed that LysR family members may regulate transcription by modulating the DNA breathing in the A+T-rich regions, allowing for formation of open complexes (33).

The transcriptional start site of *chvE* was determined by primer extension analysis (Fig. 2). Since *chvE* and *gbpR* are chromosomally encoded and therefore present at a low copy number, we attempted to increase the copy number, and therefore the number of transcript copies, by using a strain that contained a plasmid (pSL8) into which *gbpR* and *chvE* had been cloned (Table 1). Total RNA was isolated from A348(pSL8) and hybridized to 5′-^{32}P-labeled oligonucleotides complementary to the *chvE* or *gbpR* genes which served as primers for reverse transcription. The start site for *chvE* was consistently seen as a doublet of primer extension products at positions 141 and 143 (Fig. 1 and 2). This site is preceded by the sequences AACAAT in the −10 region and TTGACA in the −35 region. In numerous experiments, we were never able to see any primer extension product for *gbpR*, possibly because of its low level of expression.

Several T-N_{11}-A sequences that match the LysR family binding site motif (16) are found in the *gbpR-chvE* intergenic region. One example is located at positions 194 to 182 (minus strand), about 50 bp upstream of the *chvE* transcriptional start site (Fig. 1). Another, in the direction of *gbpR* transcription, is at positions 207 to 219.

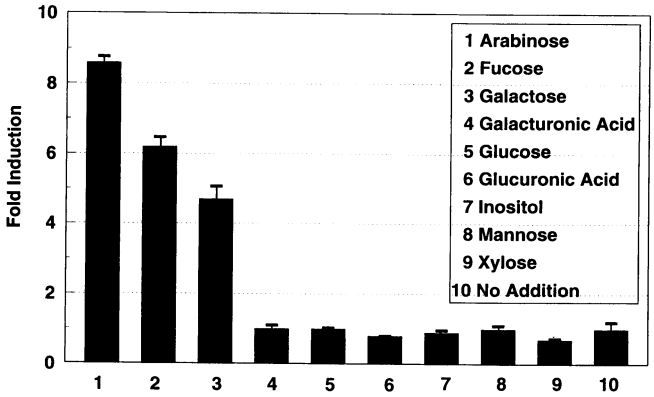

FIG. 3. Induction of *chvE::lacZ* expression by *vir* gene-inducing sugars. Strain A348(pMWH503) was grown in ABG medium containing 5 mM (each) sugar for 24 h before *chvE::lacZ* expression was measured by β-galactosidase activity. Error bars indicate the standard error in the experiment.

Effect of sugars on *chvE* expression. Since the protein encoded by *chvE* is a sugar-binding protein, it seemed reasonable to expect that its own expression would be induced by the sugars to which it binds. To test this possibility, A348 containing a *chvE::lacZ* fusion on a plasmid (pMWH503) was assayed for β-galactosidase activity when the strain was grown in nine *vir* gene-inducing sugars (5 mM [each]) or in glycerol alone, which does not induce (1). Of these, L-(+)-arabinose, D-(+)-fucose, and D-(+)-galactose induced *chvE::lacZ* expression eight-, six-, and fivefold, respectively (Fig. 3). The other six sugars did not affect the level of *chvE::lacZ* expression. Sugar concentrations of 10 and 5 mM gave approximately the same level of *chvE::lacZ* expression (data not shown). Similar results were obtained with the strain MX503, which contains a chromosomally encoded *chvE::lacZ* fusion (data not shown).

Expression of *chvE* in *gbpR* mutants. On the basis of its sequence similarity to the LysR family of transcriptional regulators and its position relative to *chvE*, it seemed likely that GbpR regulates *chvE* expression. Accordingly, *gbpR* mutants were generated to be tested for their effect on *chvE* expression. Two different *gbpR* mutants were constructed. At12001 contains a kanamycin resistance cartridge within *gbpR*. A fragment of the *gbpR* gene containing the sequence encoding the putative helix-turn-helix motif for DNA binding was deleted in At12002. This fragment was replaced with the *npt*II gene of Tn*5* (2) (see Materials and Methods and Fig. 4).

To analyze the effect of the mutant *gbpR* gene on the expression of *chvE*, a plasmid with a *chvE::lacZ* fusion but lacking a copy of *gbpR* was constructed (see Materials and Methods). pSL25/$chvE_{101}$::Tn*3*-HoHo1 was introduced into the parental strain A348 and the two *gbpR* mutant strains At12001 and At12002. *chvE::lacZ* expression was determined with cells grown in medium with or without 10 mM L-arabinose. The results of one representative experiment are shown in Fig. 5. In the strain carrying a functional *gbpR* locus, L-arabinose induced *chvE::lacZ* expression fourfold, whereas in the *gbpR* mutant strains, *chvE::lacZ* expression was not induced. We conclude that the GbpR protein acts in *trans* and is required for induction of *chvE* expression by sugars. Furthermore, in the *gbpR* mutants, the level of *chvE::lacZ* expression in the absence of sugars increased to a level which is intermediate between the basal and induced levels in the wild-type strain. This suggests that GbpR represses *chvE*

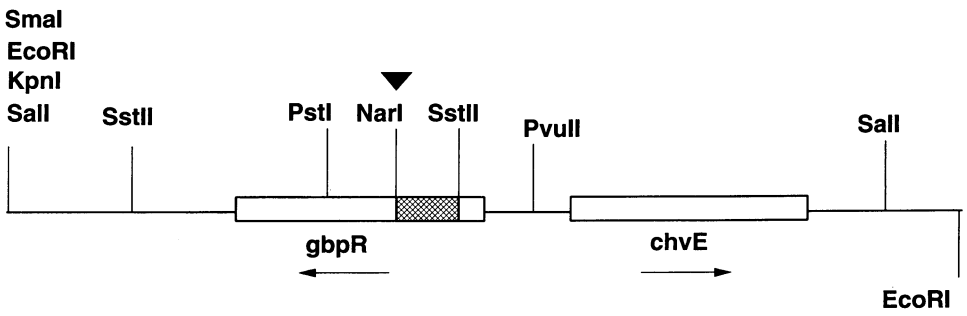

FIG. 4. Restriction map of the *gbpR-chvE* region. Arrows indicate the direction of transcription. The inverted solid triangle indicates the site of insertion of the kanamycin resistance cartridge in At12001. The crosshatched portion indicates the deletion generated in At12002.

expression in the absence of inducing sugar and activates expression in the presence of an inducer.

To verify that this phenotype was due to the absence of a functional copy of *gbpR*, a plasmid containing a wild-type *gbpR* gene (pTC116) or the vector control (pTC110) was introduced into A348, At12001, and At12002, each containing pSL25/$chvE_{101}$::Tn*3*-HoHo1. Both the repression in the absence of sugar and the induction in the presence of sugar were fully restored when a wild-type copy of *gbpR* was present (Fig. 6).

Regulation of *gbpR* expression. To determine whether *gbpR* expression is affected by sugars, we assayed *gbpR* expression in *A. tumefaciens* MX550, which carries a *gbpR*::*lacZ* chromosomal fusion. MX550 showed no induction of *gbpR*::*lacZ* expression in response to any of the nine sugars tested (data not shown). Like other LysR family members, *gbpR* appears to be expressed at a low constitutive level.

By analogy with other LysR family members, it is likely that *gbpR* is autoregulated. To determine whether GbpR does regulate its own expression, a multi-copy-number plasmid containing a wild-type copy of *gbpR* (pSL7) was introduced into MX550 and β-galactosidase activity was measured. Expression of *gbpR*::*lacZ* in MX550 containing pSL7 was reduced 27-fold relative to *gbpR*::*lacZ* expression in MX550 containing the vector alone (Fig. 7). This reduction in gene expression was unaffected by L-arabinose or D-fucose (data not shown). One explanation for these data is that *gbpR* is negatively autoregulated. An alternative explanation is that a factor necessary for *gbpR* expression was diluted out when the wild-type *gbpR* locus was present on a multi-copy-number plasmid (pSL7). To test this second possibility, a plasmid containing the *gbpR*::*lacZ* fusion (pMWH550) was introduced into either the parental strain A348 or the *gbpR* mutant strain At12002. A348 (pMWH550) and At12002(pMWH550) contain the same number of *gbpR* promoters, the only difference being that the latter strain lacks a functional *gbpR* gene. If a positive-acting factor is necessary, expression of *gbpR*::*lacZ* should be the same in both strains. A single chromosomal copy of $gbpR^+$ reduces the expression of the plasmid-encoded *gbpR*::*lacZ* fusion nearly fourfold (Fig. 7). We conclude that GbpR negatively regulates its own expression.

DISCUSSION

We have shown that the LysR family member GbpR regulates the expression of the *Agrobacterium* chromosomal virulence gene *chvE*. Our results show that *chvE* expression is induced by L-arabinose, D-fucose, and D-galactose and that this induction requires the regulatory locus *gbpR*. Presumably, the sugar interacts with the GbpR protein, which then activates

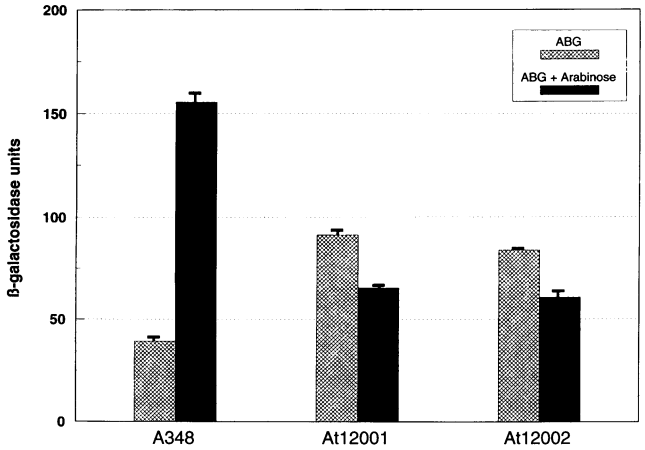

FIG. 5. *chvE* expression in parental and *gbpR* mutant strains. Strains A348, A12001, and A12002, containing pSL25/$chvE_{101}$::Tn*3*-HoHo1 (*chvE*::*lacZ*), were grown for 24 h in ABG medium with and without 10 mM L-arabinose before *chvE*::*lacZ* expression was measured by β-galactosidase activity.

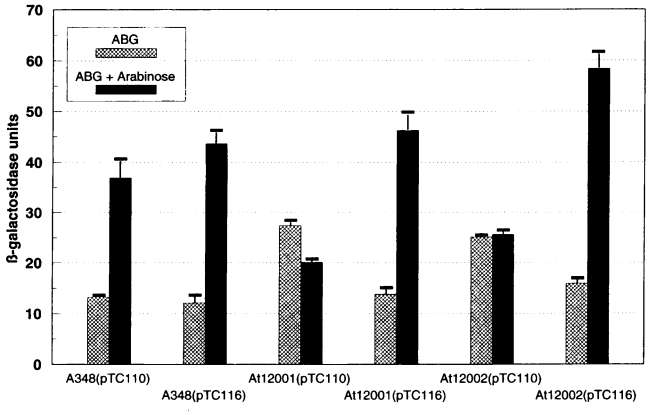

FIG. 6. Complementation of *gbpR* mutants. Strains A348, At12001, and At12002, each carrying pSL25/$chvE_{101}$::Tn*3*-HoHo1 and either pTC110 (vector control) or pTC116 ($gbpR^+$), were grown with and without 10 mM L-arabinose. *chvE*::*lacZ* expression was measured by β-galactosidase activity.

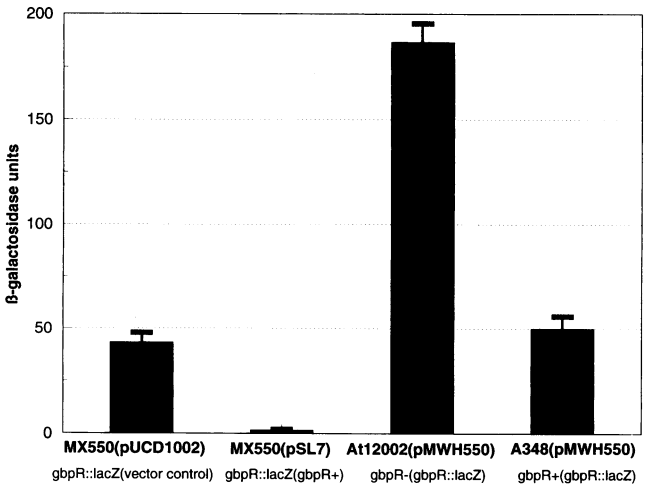

FIG. 7. *gbpR* autoregulation. MX550 (*gbpR*::*lacZ*), carrying either pSL7 (*gbpR*$^+$) or pUCD1002 (vector control), and A348(pMWH550) and A12002(pMWH550) were grown in ABG medium for 24 h. *gbpR*::*lacZ* expression was measured by β-galactosidase activity. *gbpR*$^-$, *gbpR* mutant.

expression of *chvE*. In the absence of an inducing sugar, GbpR represses *chvE* expression; in the presence of an inducing sugar, GbpR activates expression. Nearly all of the other LysR family members are only activators, although a few are only repressors (26, 33). Only a few have been shown to both repress and activate the same gene. One such family member is the AmpR protein of *Citrobacter freundii*, which regulates the expression of the β-lactamase gene. AmpR represses *ampC* expression 2.5-fold in the absence of β-lactams and induces it 11-fold in the presence of β-lactams (23). Another well-studied LysR family member that is both an activator and a repressor of the same gene is OccR (34), which regulates the expression of the octopine catabolism genes of *A. tumefaciens* in response to octopine (18). OccR binds to a single site in the overlapping promoters of *occQ* and *occR* and bends the DNA. This bend is partially relaxed when the inducer octopine is present.

The intergenic region between *gbpR* and *chvE* is nearly 350 bp long. The translational start sites of LysR family member genes and the divergently transcribed genes that they regulate are usually about 150 bp apart. This allows the promoter regions of the two genes to overlap, thus allowing regulation of both genes from one location. In order to regulate both genes, GbpR may need to bind to the two promoters separately. Alternatively, the transcriptional start sites of the two genes might be far enough from the translational start sites to allow the promoters to overlap. Our finding that *chvE* transcription begins nearly 150 bp upstream of the start codon suggests that the latter possibility is more likely.

Goethals et al. have described the presence of a T-N_{11}-A DNA binding motif in the promoters of several LysR family-regulated genes (16). Such motifs are generally 50 to 60 bp upstream of the transcriptional start site and have been shown in some cases to be the binding site for the regulator protein (16). Several T-N_{11}-A sequences occur in the *gbpR-chvE* intergenic region. Whether these serve as the sites for GbpR binding or are only present because of the A+T-rich nature of the intergenic region is unclear at this time.

Ankenbauer et al. presented data on the relative affinities of 11 inducing sugars for the ChvE protein on the basis of the concentration of the sugar which activated *vir* gene induction when low levels of acetosyringone were present (1). If *chvE* expression is itself induced to a greater degree by the most potent *vir* gene-inducing sugars, the increased quantity of ChvE protein rather than its higher affinity for the sugar could determine the effectiveness of a sugar as a *vir* gene inducer. Since we found that *chvE* expression was not induced by a subset of the sugars that induce the *vir* genes, the ChvE-sugar complex rather than the quantity of ChvE protein is important for activating VirA. Ankenbauer et al. found that induction of the virulence genes is much more sensitive to galacturonic acid than to the other 11 inducing sugars, suggesting that ChvE has the highest affinity for this sugar (1). The lack of induction of *chvE* expression by galacturonic acid supports this suggestion.

Another conclusion can be drawn from the fact that *chvE* expression is induced by only a subset of the *vir* gene-inducing monosaccharides. The GbpR protein, or a different protein if there is an indirect interaction, must have a different sugar-binding pocket than the ChvE protein. An analysis of the structures of L-arabinose, D-galactose, and D-fucose compared with the structures of the monosaccharides which did not induce *chvE* expression suggests the following structural requirements. The sugar must have a six-membered pyranose ring, equatorial hydroxyls at positions C-3 and C-4, an axial hydroxyl at C-2, and an open anomeric C-1 hydroxyl. The equatorial substitution off C-5 can be a hydrogen (L-arabinose), a methyl group (D-fucose), or a hydroxymethyl group (D-galactose).

The function of the *gbpR* gene product does not appear to be essential for any process that requires the ChvE protein (data not shown). These include growth and chemotaxis towards certain sugars, *vir* gene induction in response to sugars, and virulence on some species of plants. The level of ChvE protein in *gbpR* mutants appears sufficient to interact with the chemotaxis and uptake systems. Since it is thought that only a few molecules of the sensor protein VirA are present in the inner membrane, the elevated basal level of *chvE* expression in *gbpR* mutants presumably provides a sufficient level of ChvE protein to successfully interact with VirA to promote *vir* gene induction. In nature, where *Agrobacterium* cells may be in contact with very low concentrations of signal molecules and must move toward a plant wound site to contact plant cells, increased expression of *chvE* via GbpR might be necessary for full virulence. A *gbpR* mutant that lacked activating ability but retained repressor function could be used to determine whether it is the elevated level of *chvE* expression in *gbpR* knock-out mutants that results in the parental phenotype.

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REFERENCES

1. **Ankenbauer, R. G., and E. W. Nester.** 1990. Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. J. Bacteriol. **172:** 6442–6446.
2. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact location of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:**327–336.

2a. **Cangelosi, G. A.** Unpublished observations.

3. **Cangelosi, G. A., R. G. Ankenbauer, and E. W. Nester.** 1990.

Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA **87**:6708–6712.
4. **Cangelosi, G. A., E. A. Best, G. Martinetti, and E. W. Nester.** 1991. Genetic analysis of *Agrobacterium*. Methods Enzymol. **204**:384–397.
5. **Charles, T. C., S. L. Doty, and E. W. Nester.** Unpublished data.
6. **Charles, T. C., S. Jin, and E. W. Nester.** 1992. Two-component sensory transduction systems in phytobacteria. Annu. Rev. Phytopathol. **30**:463–484.
7. **Chilton, M.-D., T. Currier, S. Farrand, A. Bendich, M. P. Gordon, and E. W. Nester.** 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc. Natl. Acad. Sci. USA **71**:3672–3676.
8. **Cornish, A., J. A. Greenwood, and C. W. Jones.** 1988. The relationship between glucose transport and the production of succinoglucan exopolysaccharide by *Agrobacterium radiobacter*. J. Gen. Microbiol. **134**:3111–3122.
9. **Cornish, A., J. A. Greenwood, and C. W. Jones.** 1989. Binding-protein-dependent sugar transport by *Agrobacterium radiobacter* and *A. tumefaciens* grown in continuous culture. J. Gen. Microbiol. **135**:3001–3013.
10. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
11. **Farrand, S. K., S. P. O'Morchae, and J. McCutchan.** 1989. Construction of an *Agrobacterium tumefaciens* C58 *recA* mutant. J. Bacteriol. **171**:5314–5321.
12. **Feyter, R. D., Y. Yang, and D. W. Gabriel.** 1993. Gene for gene interactions between cotton R genes and *Xanthomonas campestris pv malvacearum avr* genes. Mol. Plant-Microbe Interact. **6**:225–237.
13. **Finan, T. M., B. Kunkel, G. F. De Vos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**:66–72.
14. **Gallie, D. R., S. Novak, and C. I. Kado.** 1985. Novel high- and low-copy stable cosmids for use in *Agrobacterium* and *Rhizobium*. Plasmid **14**:171–175.
15. **Garfinkel, D. J., and E. W. Nester.** 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. **144**:732–743.
16. **Goethals, K., M. Van Montagu, and M. Holsters.** 1992. Conserved motifs in a divergent *nod* box of *Azorhizobium caulinodans* ORS571 reveal a common structure in promoters regulated by LysR-type proteins. Proc. Natl. Acad. Sci. USA **89**:1646–1650.
17. **Gottfert, M.** 1993. Regulation and function of rhizobial nodulation genes. FEMS Microbiol. Rev. **10**:39–63.
18. **Habeeb, L. F., L. Wang, and S. C. Winans.** 1991. Transcription of the octopine catabolism operon of the *Agrobacterium* tumor-inducing plasmid pTiA6 is activated by a LysR-type regulatory protein. Mol. Plant-Microbe Interact. **4**:379–385.
19. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85**:6602–6606.
20. **Huang, M.-L. W., G. A. Cangelosi, W. Halperin, and E. W. Nester.** 1990. A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. J. Bacteriol. **172**:1814–1822.
21. **Jin, S., R. K. Prusti, T. Roitsch, R. G. Ankenbauer, and E. W. Nester.** 1990. Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. J. Bacteriol. **172**:4945–4950.
22. **Knauf, V. C., and E. W. Nester.** 1982. Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid **8**:45–54.
23. **Lindberg, F., L. Westman, and S. Normark.** 1985. Regulatory components in *Citrobacter freundii ampC* β-lactamase induction. Proc. Natl. Acad. Sci. USA **82**:4620–4624.
24. **MacNab, R. M.** 1987. Motility and chemotaxis, p. 732–759. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
25. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 21. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Neidle, E. L., C. Hartnett, and L. N. Ornston.** 1989. Characterization of *Acinetobacter calcoaceticus catM*, a repressor gene homologous in sequence to transcriptional activator genes. J. Bacteriol. **171**:5410–5421.
27. **Nunn, D., S. Bergman, and S. Lory.** 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172**:2911–2919.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
29. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn3 *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**:891–898.
30. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
31. **Summers, W. C.** 1970. A simple method for extraction of RNA from *E. coli* utilizing diethylpyrocarbonate. Anal. Biochem. **33**: 459–463.
32. **Triezenberg, S.** 1991. Primer extension, p. 4.8.1.–4.8.4. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley and Sons, Inc., New York.
33. **Viale, A. M., H. Kobayashi, T. Akazawa, and S. Henikoff.** 1991. *rcbR*, a gene coding for a member of the LysR family of transcriptional regulators, is located upstream of the expressed set of ribulose 1,5-bisphosphate carboxylase/oxygenase genes in the photosynthetic bacterium *Chromatium vinosum*. J. Bacteriol. **173**: 5224–5229.
34. **Wang, L., J. D. Helmann, and S. C. Winans.** 1992. The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell **69**:659–667.
35. **Winans, S. C.** 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. **56**:12–31.
36. **Winans, S. C., P. R. Ebert, S. E. Stachel, M. P. Gordon, and E. W. Nester.** 1986. A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. Proc. Natl. Acad. Sci. USA **83**:8278–8282.
37. **Zambryski, P., J. Tempe, and J. Schell.** 1989. Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. Cell **56**:193–201.