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Nucleotide sequence of the virulence gene *vir*G of the *Agrobacterium tumefaciens* octopine Ti plasmid: significant homology between *vir*G and the regulatory genes *omp*R, *pho*B and *dye* of *E. coli*

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ABSTRACT

The entire nucleotide sequence of the virG locus, from the octopine Ti plasmid of Agrobacterium tumefaciens strain 15955, has been determined. The virG gene is 801 nucleotides in length and has one open reading frame which encodes a protein of Mr 29,995. The virG gene is involved in the transcriptional activation of the Ti plasmid vir-loci, which occurs after induction by specific compounds present in plant exudate. Sequence analysis of the Agrobacterium virG protein showed significant homology with the Escherichia coli ompR, phoB and dye proteins, which are all positive regulatory genes for genes encoding envelope proteins. These results suggest that the virG gene encodes a positive regulatory protein which can activate vir gene expression.

INTRODUCTION

Agrobacterium induced tumor formation is the result of genetic transformation of plant cells at infection sites of wounded dicotyledonous plants. During transformation the bacterium transfers a specific part of the Ti plasmid (the T-region) into the plant genome where it becomes stably integrated (1-3). The T-region encodes enzymes which are directly involved in auxin and cytokinin biosynthesis (4-6) and other which mediate the production of opines (7,8). In the Ti plasmid the T-region is flanked by identical 24 bp direct repeats (9-11). The DNA between the T-region border repeats can be fully deleted without affecting its transfer to plant cells (12). Thus, the genes of the T-region are responsible for tumor induction, but not for gene transfer itself. The process of T-region transfer is mediated by the genes of the Ti plasmid virulence (Vir)-region which contains at least seven operons and encodes trans-acting products (13-17). Some vir-loci are absolutely essential (virA, virB, virD and virG) for tumor induction while other loci (virC, virE, virF) are only necessary for tumor induction on certain plant species. The functions encoded by genes in the Vir-region are not known,

but some proteins encoded by the vir-genes probably mediate T-region processing and transfer. Expression of Agrobacterium vir-genes is specifically induced by factors present in the exudates of wounded plant cells (18,19). The transcriptional activation of the virulence genes is proposed to be a primary event in the complex process of gene transfer to plant cells. Recently, a number of phenolic compounds were identified as the active plant signal molecules (20,21). Activation of the vir regulatory system can be accomplished by transduction of the plant signal to either positive or negative regulatory proteins that interact with vir promoter sequences or to Agrobacterium RNA polymerase itself. The regulation of the vir induction process is not known in detail but recent data suggest that the genes virA and virG are involved in the induction process (22).

In this work, we studied the nucleotide sequence of the virG gene of the octopine type plasmid pTi15955. The amino acid sequence of the virG protein, deduced from the DNA sequence, revealed extensive homology with those of the ompR, dye and phoB proteins, which are positive regulators for several genes encoding E.coli envelope proteins (23-25).

MATERIALS AND METHODS

Bacterial strains

Strain JM101 (supE, thi, Δ(lac-proAB) / F', traD36, proAB, $lacI^{q}Z\Delta M15$) was used as the E.coli host for plasmid constructs and was grown in LC-medium (26). Agrobacterium tumefaciens strain15955 (LBA 8255) was grown in minimal (27) or LC-medium.

Enzymes and Chemicals

Restriction endonucleases were purchased from either Promega Biotec or New England Biolabs. T4 polynucleotide kinase was purchased from Pharmacia PL Biochemicals. (γ-^{32}P)ATP was purchased from New England Nuclear.

DNA sequencing

DNA sequence reactions were conducted according to the method of Maxam and Gilbert (28), as modified by Barker et al. (11). Sequence data was analyzed using the programs of Martinez and Devereux et al. (29).

DNA isolation and plasmid constructs

Plasmid DNA was isolated from E.coli as described by Birnboim and Doly (30). From A.tumefaciens Ti plasmid DNA was isolated according to Koekman et al. (31). Plasmid pRAL3230 contains the pTi15955 fragments HindIII-3;37b (Fig.1) cloned into pUC19. The restriction fragments

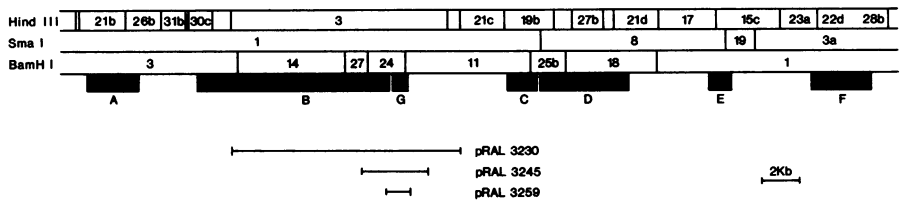

Figure 1. Physical map of the octopine plasmid pTi15955 virulence region. Map positions of the seven different vir-loci are shown. Three vir clones, plasmids pRAL3230, pRAL3245 and pRAL3259, are shown below as horizontal lines.

SalI-13b and BamHI-24 in pRAL3230 were subcloned in pUC19 and were designated pRAL 3245 and pRAL3232 respectively. The 1.25 kb BglII-PstI restriction fragment carrying the virG locus was isolated from pRAL3245 and cloned in vector pIC19R to yield pRAL3259.

RESULTS

Cloning and sequencing the virG locus

The Vir-region of the octopine Ti plasmid, which is approximately 40 kb in size, contains genes essential for virulence of Agrobacterium. Genetic analysis of this region has shown the presence of seven vir-complementation groups, virA, virB, virC, virD, virE, virF and virG (Fig.1) (13,14,16,17). According to the physical map of the Ti Vir-region virG is present on a pTi15955 HindIII-3 fragment which was cloned into the pUC19 vector (33). Further subcloning resulted in the isolation of vir-clones pRAL3245 and pRAL3259 (see Fig.1). Genetic analysis showed that the virG locus was present on a 1.25 kb BglII-PstI fragment (Melchers et al. unpublished; 22). The DNA sequence of this 1.25 kb BglII-PstI fragment encompassing the virG coding region was determined using the method of Maxam & Gilbert (28). The sequencing strategy is shown in figure 2 and the

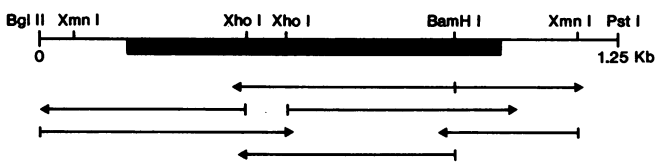

Figure 2. Partial restriction map and strategy of sequencing the virG gene. Sequencing was carried out as described in the methods section. Only those restriction sites used for sequencing are presented. The arrows indicate the extent and direction of DNA sequencing. The thick line corresponds to the coding region.

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   1 CGAGATCTGGCTCGCGGCGGACGCACGACGCCGGGGCGAGACCATAGGCGATCTCCTAAATCAATAGTAGCTGTAACC  78

  79 TCGAAGCGTTTCACTTGTAACAACGATTGAGAATTTTTGTCATAAAATTGAAATACTTGGTTCGCATTTTTGTCATCC 156

 157 GCGGTCAGCCGCAATTCTGACGAACTGCCCATTTAGCTGGAGATGATTGTACATCCTTCACGTGAAAATTTCTCAAGC 234
                                                MetIleValHisProSerArgGluAsnPheSerSer

 235 GCTGTGAACAAGGGTTCAGATTTTAGATTGAAAGGTGAGCCGTTGAAACACGTTCTTCTTGTCGATGACGACGTCGCT 312
     AlaValAsnLysGlySerAspPheArgLeuLysGlyGluProLeuLysHisValLeuValAlaAspAspAspValAla

 313 ATGCGGCATCTTATTATTGAATACCTTACGATCCACGCCTTCAAAGTGACCGCGGTAGCCGACAGCACCCAGTTCACA 390
     MetArgHisLeuIleIleGluTyrLeuThrIleHisAlaPheLysValThrAlaValAlaAspSerThrGlnPheThr

 391 AGAGTACTCTCTTCCGCGACGGTCGATGTCGTGGTTGTTGATCTAAATTTAGGTCGTGAAGATGGGCTCGAGATCGTT 468
     ArgValLeuSerSerAlaThrValAspValValValValAspLeuAsnLeuGlyArgGluAspGlyLeuGluIleVal

 469 CGTAATCTGGCGGCAAAGTCTGATATTCCAATCATAATTATCAGTGGCGACCGCCTTGAGGAGACGGATAAAGTTGTT 546
     ArgAsnLeuAlaAlaLysSerAspIleProIleIleIleIleSerGlyAspArgLeuGluGluThrAspLysValVal

 547 GCACTCGAGCTAGGAGCAAGTGATTTTATCGCTAAGCCGTTCAGTATCAGAGAGTTTCTAGCACGCATTCGGGTTGCC 624
     AlaLeuGluLeuGlyAlaSerAspPheIleAlaLysProPheSerIleArgGluPheLeuAlaArgIleArgValAla

 625 TTGCGCGTGCGCCCCAACGTTGTCCGCTCCAAAGACCGACGGTCTTTTTGTTTTACTGACTGGACACTTAATCTCAGG 702
     LeuArgValArgProAsnValValArgSerLysAspArgArgSerPheCysPheThrAspTrpThrLeuAsnLeuArg

 703 CAACGTCGCTTGATGTCCGAAGCTGGCGGTGAGGTGAAACTTACGGCAGGTGAGTTCAATCTTCTCCTCGCGTTTTTA 780
     GlnArgArgLeuMetSerGluAlaGlyGlyGluValLysLeuThrAlaGlyGluPheAsnLeuLeuLeuAlaPheLeu

 781 GAGAAACCCCGCGACGTTCTATCGCGCGAGCAACTTCTCATTGCCAGTCGAGTACGCGACGAGGAGGTTTATGACAGG 858
     GluLysProArgAspValLeuSerArgGluGlnLeuLeuIleAlaSerArgValArgAspGluGluValTyrAspArg

 859 AGTATAGATGTTCTCATTTTGAGGCTGCGCCGCAAACTTGAGGCGGATCCGTCAAGCCCTCAACTGATAAAAACAGCA 936
     SerIleAspValLeuIleLeuArgLeuArgArgLysLeuGluAlaAspProSerSerProGlnLeuIleLysThrAla

 937 AGAGGTGCCGGTTATTTCTTTGACGCGGACGTGCAGGTTTCGCACGGGGGGACGATGGCAGCCTGAGCCAATTGCATT 1014
     ArgGlyAlaGlyTyrPhePheAspAlaAspValGlnValSerHisGlyGlyThrMetAlaAlaEnd

1015 TGCCTCTTAATTATCTGGCTCAAAGGGTGACTGAGGAGTAAGCGATGTGCCCATCACACTGACCACCAAGACGG 1088
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Figure 3. Nucleotide sequence of the pTi15955 virG gene. The 801 bp open reading frame, extending from nucleotide 118-918 and the derived amino acid sequence are shown. The arrows indicate the presence of a direct repeat.

sequence in figure 3. The nucleotide sequence of the virG locus contained one open reading frame, which encoded a polypeptide of Mr 29,955. Analysis of the 5'-end promoter region of the virG DNA sequence did not show plausible matches to the consensus sequences of E.coli promoters (34). The sequences preceding the putative coding region lack a sequence similar to the Shine-Dalgarno ribosome binding site found in E.coli (35). The direction of transcription of the octopine pTi15955 virG coding region was clockwise towards the T-region which agreed with the transcription direction of the octopine pTiA6 virG locus as determined by Stachel et al. (17). The nucleotide sequences of the promoter regions and

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      1                                          * *·*·   *  .   . .      50
virg.gap      MIVHPSRENF SSAVNKGSDF RLKGEPLKHV LLVDDDVAMR HLIIEYLTIH
ompr.gap      .......... .......... ...MQENYKI LVVDDDMRLR ALLERYLTEQ
phob.gap      .......... .......... .....MARRI LVVEDEAPIR EMVCFVLEQN
Consensus     ---------- ---------- ---------- l-v-d----r ------l---

     51         .  ...   .  . *      .  . *·  *  ·-*·. .  . *          100
virg.gap      AFKVTAVADS TQFTRVLSSA TVDVVVVDLN LGREDGLEIV RNLAAKSDIP
ompr.gap      GFQVRSVANA EQMDRLLTRE SFHLMVLDLM LPGEDGLSIC RRLRSQSNPM
phob.gap      GFQPVEAEDY DSAVNQLNEP WPDLILLDWM LPGGSGIQFI KHLKRESMTR
Consensus     -f-------- ------l--- -------d-- l----g---- --l---s---

     101             .  .. . . . . .. . . . ...  .  .* .    .          150
virg.gap      II..IISGDR LEETDKVVAL ELGASDFIAK PFSIREFLAR IRVALRVRPN
ompr.gap      PIIMVTAKGE EVDRIVGLEI GADDYIPKPF NPRELLARIR AVLRRQANEL
phob.gap      DIPVVMLTAR GEEEDRVRGL ETGADDYITK PFSPKELVAR IKAVMRRISP
Consensus     -i-------- ---------- ---------- ---------r ----------

     151                          .     .          .. .   . ..   .      200
virg.gap      VVRSKDRRSF CFTDWTLNLR QRRLMSEAGG EVKLTAGEFN LLLAFLEKPR
ompr.gap      PGAPSQEEAV IAFGKFKLNL GTREMFREDE PMPLTSGEFA VLKALVSHPR
phob.gap      MAVEEVIEMQ GLSLDPTSHR ....VMAGEE PLEMGPTEFK LLHFFMTHPE
Consensus     ---------- ---------- ---------- -------ef- -l------p-

     201       ..  . . .    . .   .  . .. *      *. .  ..    .  . . .  .      250
virg.gap      DVLSREQLLI ASRVRDEEVY DRSIDVLILR LRRKLEADPS SPQLIKTARG
ompr.gap      EPLSRDKLMN LARGREYSAM ERSIDVQISR LRRMVEEDPA HPRYIQTVWG
phob.gap      RVYSREQLLN HVWGTNVYVE DRTVDVHIRR LRKALEPGGH DRMVQTVRGT
Consensus     ---sr--l-- ---------- -r--dv-i-r lr---e---- ----------

     251       .... .                                                  269
virg.gap      AGYFFDADVQ VSHGGTMAA
ompr.gap      LGYVFVPDGS KA.......
phob.gap      GYRFSTRF.. .........
Consensus     ---------- ---------
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Figure 4. Comparison of the amino acid sequences of the virG, ompR and phoB proteins. The three proteins are aligned for best fit utilizing gaps. Identical amino acids found in all of 3 proteins are shown in the consensus sequence. Conserved amino acids also present in the dye protein are marked with a star (*). A dot in the upper line indicates positions at which virG shares homology with one of the other aligned polypeptides. Protein sequences are derived by deduction from the DNA sequences (ref. see table I).

Table I. Characteristics of the virG, ompR, phoB and dye proteins.

protein	amino acids	Mr	homology[1] %	net charge	reference
virG	267	29,955	100.0	1	this work
ompR	239	27,353	28.9	-2	38.
phoB	229	26,433	26.6	-8	25.
dye	238	27,291	11.8	-10	24.

1. % amino acids of the protein homologous with the virG protein

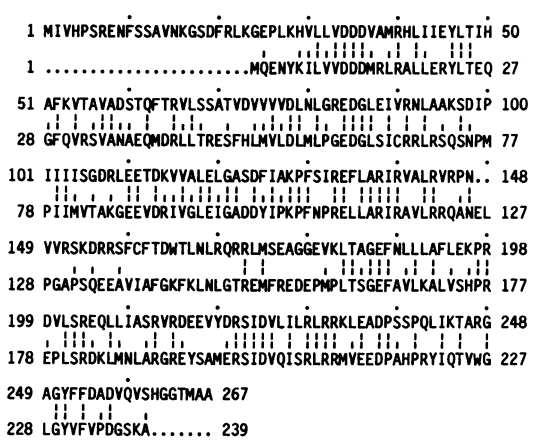

Figure 5. Comparison of virG protein and ompR protein. The upperline displays the amino acid sequence of the virG protein from pTi15955 and the lower line the amino acid sequence of the ompR protein from E.coli. Two vertical bars indicate sequence identities and one bar indicates a conservative amino acid replacement.

transcription initiation sites of six pTiA6 vir-genes have been determined. The sequence of the pTi15955 virG promoter region turned out to be identical to the virG promoter region of plasmid pTiA6 as determined by Das et al. (36).

Homology among the virG, ompR, phoB and dye proteins.

We searched for proteins with homology to the virG protein, using the NBRF protein sequence data base. Extensive homology was found with the sequence of the E.coli ompR protein as deduced from the DNA sequence (37,38). The ompR protein is a regulator for the ompF and ompC genes which code for outer membrane porin proteins (23). Significant sequence homology has already been reported for the E.coli genes called ompR, phoB and dye (24,25). The dye protein may be involved in the regulation of genes coding for various envelope proteins and genes involved in the transmission of the F plasmid of E.coli (39,40). The phoB gene encodes a positive regulator for genes belonging to the phosphate regulon of E.coli (41). We compared the amino acid sequence of the virG protein with those of the ompR, phoB and dye proteins in detail. The virG protein consisted of about 48.3% polar amino acids, a number similar to ompR (48.1%) and phoB (48.9%) but slightly lower than present in the dye protein (54.2%). Considerable homology was found between virG, ompR and phoB throughout their entire amino acid sequence. However, the virG gene product showed

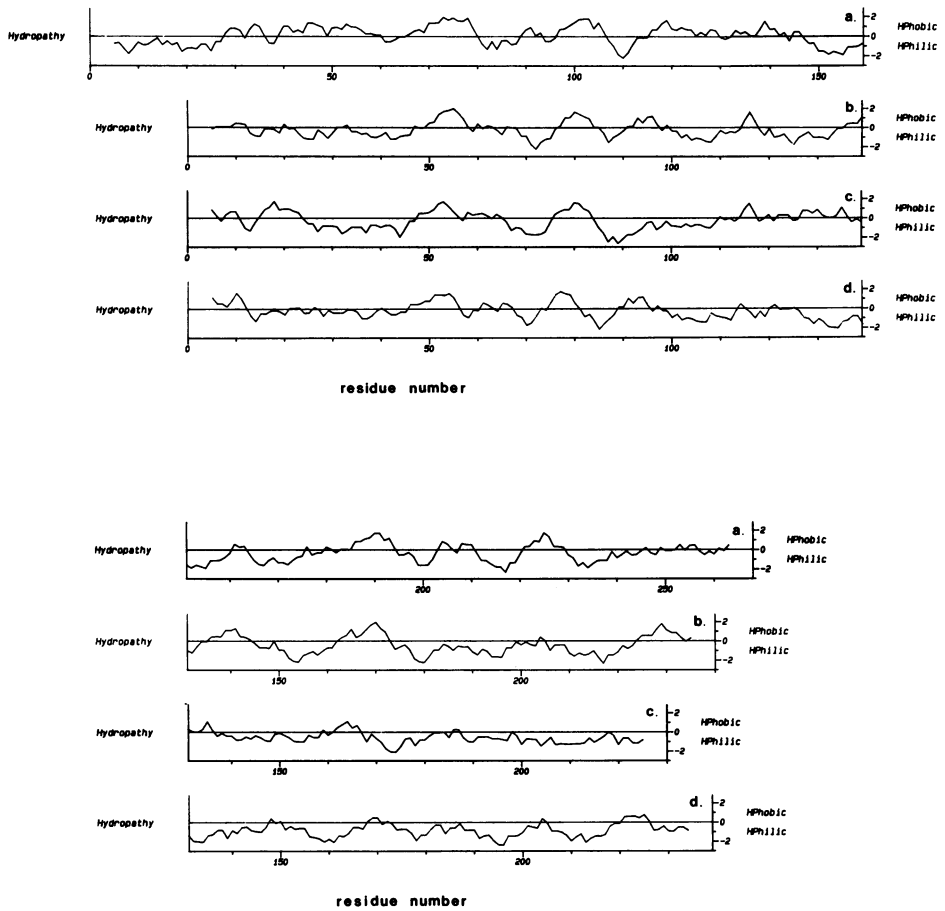

Figure 6. Comparison of hydropathy profiles between virG (a), ompR (b), phoB (c) and dye (d) proteins. The hydropathy profiles (values averaged over nine amino acids) are plotted against the amino acid sequence positions by the method of Kyte and Doolittle (32).

the highest homology with ompR and phoB proteins especially from amino acid 31-102 and amino acid 188-236 (Fig.4). There was less homology present between virG and the dye coding region (Table I). Conserved amino acids present in the proteins virG, ompR, phoB and dye are shown in figure 4. Although the number of amino acids which are conserved in all four proteins is rather low, most of the non-homologous residues are conservative amino acid replacements as shown for virG and ompR in figure 5. Considering these functionally conserved amino acid changes the

proteins ompR, phoB and dye turned out to be extensively homologous (about 60%) with the virG protein. To determine whether the four proteins still conserve similarity in the tertiary structure, we compared the distribution of hydrophobic groups along the proteins. Their hydropathy profiles were very similar even in regions where the primary structures were less homologous (Fig.6). These results suggest that the conformations of the four proteins are stereochemically very similar, whereby the observed amino acid consensus is probably essential for the common mode of action of these regulatory proteins.

DISCUSSION

Agrobacterium tumefaciens transfers its Ti-plasmid T-region to plant cells and this process is dependent on the expression of the vir-genes. Induction of vir-gene expression in Agrobacterium is a vir and Agrobacterium specific phenomenon that is mediated by an inducing factor present in exudates of dicotyledonous plant cells. It is not known how vir-induction is regulated in Agrobacterium. However the virA and virG genes turned out to be the only vir-genes involved in the activation of the vir-regulon (22).

Nucleotide sequence analysis of the virG locus, presented in this paper, revealed the presence of a single open reading frame of 267 codons that codes for a protein of Mr29,955. The virG protein turned out to be closely related to a number of positive regulatory proteins viz. the ompR, phoB and dye proteins from E.coli. The significant homology of the virG protein to these E.coli regulatory proteins, is in agreement with its regulatory role in vir expression and suggests that virG might function in a similar way as the above mentioned proteins. The E.coli genes ompR, phoB and dye, probably evolved from a protogene, have different but still related functions as regulators for the genes coding for envelope proteins. Although the function of the virG gene is unknown, it is possible that virG protein has an analogous function as ompR, phoB and dye in regulation of cell surface components. The alteration of cell surface components is likely to be an essential event in the transfer of the T-region across the cell wall of the bacterium to the plant cell.

The E.coli genes ompR and envZ constitute an operon, while phoB forms an operon with phoR. Genetic analysis has shown that envZ and phoR encode membrane proteins which are essential for the activation of the omp and pho-genes respectively. The envZ protein is an envelope protein which

senses the external environment and transduces a cytoplasmic signal to the ompR protein by which this becomes activated (23). In view of the homology between ompR and virG it is tempting to speculate on the possible presence of an envZ like gene in Agrobacterium. In fact by sequence analysis we and others found recently that the virA gene of the Ti plasmid determines a membrane protein with gross structural homology to envZ (42-44). Therefore the virA protein potentially functions in the initial recognition of the plant signal molecule and transduces or transports this signal to activate the virG protein. Upon activation, the virG protein in turn activates transcription of the vir-regulon.

The knowledge of the virG DNA sequence will allow further study of the virG protein, particularly with respect to its regulatory function in vir gene expression.

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