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The binding site of the transcriptional activator VirG from *Agrobacterium* comprises both conserved and specific nonconserved sequences

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Abstract

Virulence genes of *Agrobacterium tumefaciens* are transcriptionally activated in response to phenolic compounds and certain sugars. The transcriptional activator VirG specifically binds to fragments containing the conserved *vir box* sequence present in the promoter region of all *vir* genes. This study shows that both the *vir box* as well as specific nonconserved sequences downstream of the *vir box* are required for VirG binding and transcriptional activation. Insertion of the identified VirG binding site into the *lac* promoter resulted in transcriptional activation of this heterologous promoter in response to the plant phenolic signal molecule acetosyringone.

Key words: Agrobacterium; Virulence; *vir* gene induction; VirG

1. Introduction

Agrobacterium tumefaciens causes crown gall disease on a wide variety of dicotyledonous and some monocotyledonous plants by the transfer of procaryotic DNA (T-DNA) from a large tumor-inducing (Ti) plasmid to the plant genome (reviewed in [1]). The infection process requires the expression of genes located both on the bacterial chromosome (*chv* genes) and on the Ti-plasmid (*vir* genes). The *vir* genes are transcriptionally activated in response to phenolic compounds such as acetosyringone [2] and certain sugars which are released by wounded plant tissue [3,4].

Two of the *vir* genes, *virA* and *virG*, are required for the expression of all of the *vir* genes [5]. Previous studies have demonstrated that the corresponding gene products are members of a family of two component regulatory systems which regulate the response of bacterial cells to their environment via a cascade of phosphorylation reactions (reviewed in [6]). The sensor protein VirA acts as a kinase [7,8] whereas VirG functions as a transcriptional regulator of the *vir* genes similar to most of the response regulator proteins [9].

The *Agrobacterium vir* genes are characterized by one or more conserved dodecadeoxynucleotide sequences (*vir box*) in the promoter region [10]. At least one of the *vir boxes* of each operon is required for transcriptional activation in response to acetosyringone [11–14], and the VirG protein was shown to specifically bind to fragments containing this cis acting regulatory sequence [12,15,16]. Bal31 deletion analysis revealed that sequences upstream of the *vir box* sequences could be removed without affecting induction of the *vir* genes [11, 12]

In the present study we show that the *vir box* is not sufficient for binding of the VirG protein, but requires additional specific nonconserved sequences 3′ to the *vir box*. We further show that the presence of the VirG binding site results in transcriptional activation of the *E. coli lac* promoter in response to acetosyringone.

2. Materials and methods

2.1. Bacterial strains

A. tumefaciens strain A348 is a derivative of strain A136 containing the octopine-catabolizing plasmid pTiA6 [17]. *A. tumefaciens* strains Mx321 and A1030 carry mutations in the *virG* and *virA* genes, respectively [18].

2.2. Plasmids and plasmid constructions

Plasmid pSG673 [19] contains a truncated *virA* gene under the control of the *lac* promoter and plasmid pSG675 [12] contains the *virE* gene.

The *virE vir box* (TGCAGTTGAAAC) was inserted into the *lac* promoter of plasmid pSG673 at position −54 (with respect to the transcriptional start site) by oligonucleotide directed site specific mutagenesis [20] resulting in plasmid pRS3220 (*VB:lacp*).

Fragments from the *virE* promoter containing the *vir box* plus 5 and

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Abbreviations: *lacp*, lac promoter; *VB*, *vir box* sequence; *VB/5*, *VB/19*, *VB/34*, sequence comprising the *virE vir box* and 5, 19, or 34 nucleotides 3′ to the *vir box*.

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19 additional nucleotides at their 3′ end, designated *VB/5* and *VB/19* (see Fig. 1), were subcloned into the *lac* promoter as follows. A *Sma*I restriction site was introduced into the *lac* promoter on plasmid pSG673 at position −31 by oligonucleotide directed site specific mutagenesis resulting in plasmid pRS9812. This restriction enzyme site was used to subclone a *Sma*I–*Eco*RV (about 500 bp) and a *Ava*I fragment, blunt ended with *Klenow* polymerase (about 520 bp) from plasmid pSG675 [12] to construct plasmids pRR1340 (*VB/5:lacp*) and pRS9902 (*VB/19:lacp*). The 5′ *Ava*I and *Sma*I sites used for subcloning are located within the same polylinker and thus the two fragments share identical sequences upstream of the *vir box*. The positions of the *vir box* in the modified *lac* promoter of the plasmids pRS3220 (*VB:lacp*) and pRS9902 (*VB19/lacp*) were the same as in the *virE* promoter with respect to the transcriptional start site (nucleotides −65 to −54).

Fragments were subcloned into the broad host range vector pUCD2 [21] and a fusion of the *lac* promoter constructs with the *lacZ* coding region was constructed as follows. The truncated *virA* gene under control of the native and modified *lac* promoter in plasmids pSG673, pRS3220, pRR1340 and pRS9902 were subcloned as *Pvu*II–*Asp*718 fragments into the vector pUCD2 which had been cut with the same enzymes resulting in plasmids pRS4724, pRS4602, pRR1605 and pRS10001, respectively. Fusions of the wildtype *lac* promoter and the modified *lac* promoter with the *lacZ* coding region were constructed by substituting the truncated *virA* gene for the *lacZ* coding region. The *Bgl*II–*Asp*718 fragments with the truncated *virA* gene from pRS4724, pRS4602, pRR1605 and pRS10001 were substituted for a *Bam*HI–*Asp*718 fragment from plasmid pRS8102 (a derivative of pMC1402 [22] with a unique *Asp*718 site 3′ of the *lacZ* coding region; Roitsch and Nester, unpublished), resulting in plasmids pRS8501 (*plac*), pRS8401 (*VB:plac*), pRR2001 (*VB/5:plac*) and pRS10201 (*VB/19:plac*), respectively.

The *vir box* of the *virE* promoter on plasmid pSG675 was deleted by oligonucleotide directed site specific mutagenesis resulting in plasmid pRS2013. The *virE vir box* was reinserted at the wild type position but in inverse orientation into the mutated *virE* promoter on plasmid pRS2013 by oligonucleotide directed site specific mutagenesis to construct plasmid pRS3324.

Plasmid pRS3420 was constructed by deleting 13 bp downstream of the *vir box* of the *virA* promoter on plasmid pSW169 by oligonucleotide directed site specific mutagenesis. Thus the *virA vir box* was moved to the position of the *virE vir box* with respect to the transcriptional start site.

A schematic representation of the various promoter constructs described above is shown in Fig. 2.

All mutations created by oligonucleotide directed site specific mutagenesis were confirmed by DNA sequence analysis [23].

2.3. Other methods

Protein binding assays using two complementary 12 bp oligonucleotides, TGCAATTGAAAC and GTTTCAATTGCA, representing both strands of the *vir box* consensus sequence were carried out as follows. The oligonucleotides were end labeled by *T4-DNA-kinase* using [γ-^{32}P]ATP. The two oligonucleotides were annealed by incubation of equimolar amounts at 90°C for 5 min and slowly cooling the assay to room temperature. In each binding assay 10,000 cpm of labeled oligonucleotides (5 ng) were used with different amounts of purified VirG protein overproduced in *E. coli* [12]. The conditions for binding and electrophoresis as well as the conditions for the binding assays using DNA restriction fragments from the various promoter constructs were the same as described previously [12].

Other techniques were carried out according to published procedures: These included induction assays and western (immuno-) blot analysis [24].

3. Results

3.1. Binding of VirG requires specific nonconserved sequences in addition to the conserved vir box

Previous gel retardation assays demonstrated that the transcriptional activator VirG specifically binds to *vir* gene promoter fragments and DNA footprinting analyses revealed that the conserved *vir box* sequences are specifically protected by VirG [12,15]. Since fragments with large flanking sequences were used in these studies we wanted to determine whether the *vir box* sequence alone was sufficient for binding of the regulatory protein VirG. We carried out a gel retardation experiment using two complementary 12mer oligonucleotides representing the *vir box* consensus sequence, TGCAATTGAAAC [10], as a double stranded probe. The addition of VirG did not result in a retarded band indicating that VirG did not bind to the double stranded oligonucleotide probe (not shown). Thus the *vir box* sequence lacking any flanking sequences is not sufficient for VirG binding. Since it has been shown in other systems that regulatory proteins are able to bind to oligonucleotides representing the binding sequence [25,26] this result indicated that VirG binding requires sequences in addition to the conserved *vir box*.

Bal31 deletion analysis from the *virE* [12] and *virB* [11] promoter demonstrated previously that sequences 5′ of the *vir box* could be deleted without affecting transcriptional activation. These results indicate that sequences 5′ of the *vir box* are not important for binding of the regulatory protein. Therefore we tested the binding of the VirG protein to the *virE* promoter fragments with the *vir box* and 5, 19 or 34 additional nucleotides 3′ to the *virE vir box* (*VB/5*, *VB/19*, and *VB/34*) at their 3′ end (Fig. 1). The DNA binding assay shown in Fig. 3A revealed that the VirG protein bound only to the fragments containing 19 and 34 additional nucleotides downstream of the *vir box*. This result shows that the VirG binding site of the *vir* promoter comprises both the *vir box* and additional nucleotides 3′ to the *vir box*. Using the *VB/34* fragment a better retardation is visible compared to *VB/19* with 0.2 μg VirG protein. This may be due to the fact the binding sites are located at the very 3′ end of the fragments used for this experiment. Alternatively, it may be possible that the *VB/19* sequence contains only a partial binding site whereas the *VB/34* sequence contains a complete binding site. Our previous DNA footprinting analysis using the *virE* promoter fragments revealed that not only the *vir box* but also flanking sequences were protected by VirG [12], which indicates that sequences on both sites of the *vir box* are at least in contact with virG. Therefore better

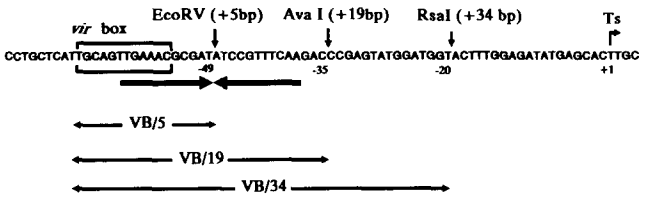

Fig. 1. Schematic representation of the promoter region of the *virE* promoter. The 3′ restriction enzyme sites used for subcloning and to generate fragments for the gel retardation experiment shown in Fig. 3A are indicated. The arrows indicate the position of a palindromic sequence; Ts: transcriptional start site.

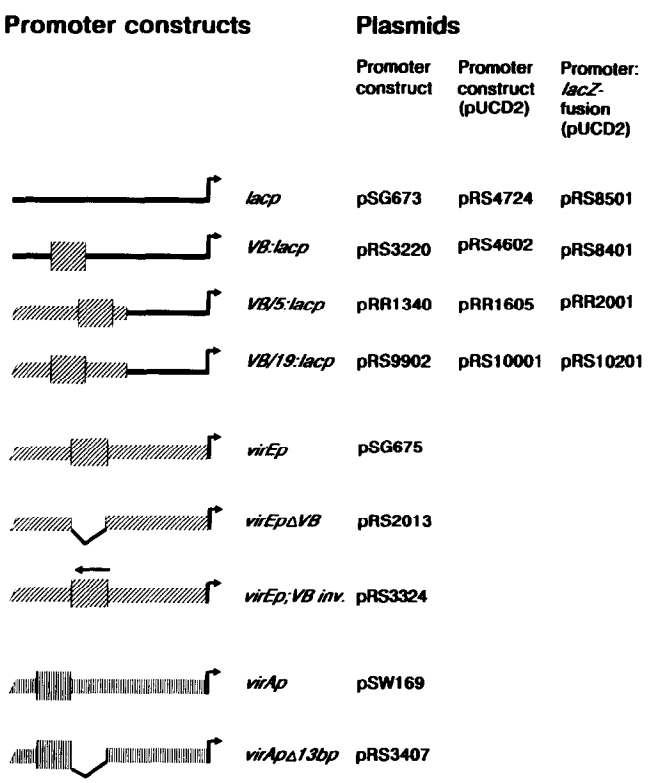

Fig. 2. Schematic representation of the various promoter constructs used in this study and the designation of the corresponding plasmid constructs. The figures are not drawn to scale.

binding to the *VB/34* fragment may be due to unspecific stabilization of the binding to the binding site located within the *VB/19* sequence.

To address the question of whether specific sequences 3′ of the *vir box* are required for VirG binding and to gain additional insight into the number of nucleotides required for binding we have introduced the 12 bp *vir box* sequence and a fragment with the *VB/19* sequence into the *lac* promoter. In each construct the *vir box* was inserted at the same position as in the *virE* promoter with respect to the transcriptional start site (nucleotides −65 to −54). Gel retardation experiments were carried out to assay the binding of VirG to DNA fragments derived from the wild type *lac* promoter and constructs with the modified *lac* promoters. Fig. 3B demonstrates that VirG specifically binds only to fragments derived from the *lac* promoter with the *VB/19* sequence. The VirG protein did not bind to the wildtype *lac* promoter nor to the *lac* promoter with the 12 bp *vir box* sequence.

These findings demonstrate that the VirG binding site has a complex structure and consists of both of the conserved *vir box* sequence and specific nonconserved sequences 3′ of the *vir box*. Thus, the *VB/19* sequence is necessary and sufficient for VirG binding and the binding site is either identical to this sequence or located within this sequence.

The result that specific sequences downstream of the *vir box* sequence are required for VirG binding and transcriptional activation of the *vir* genes was supported by additional experiments. We have constructed a mutant

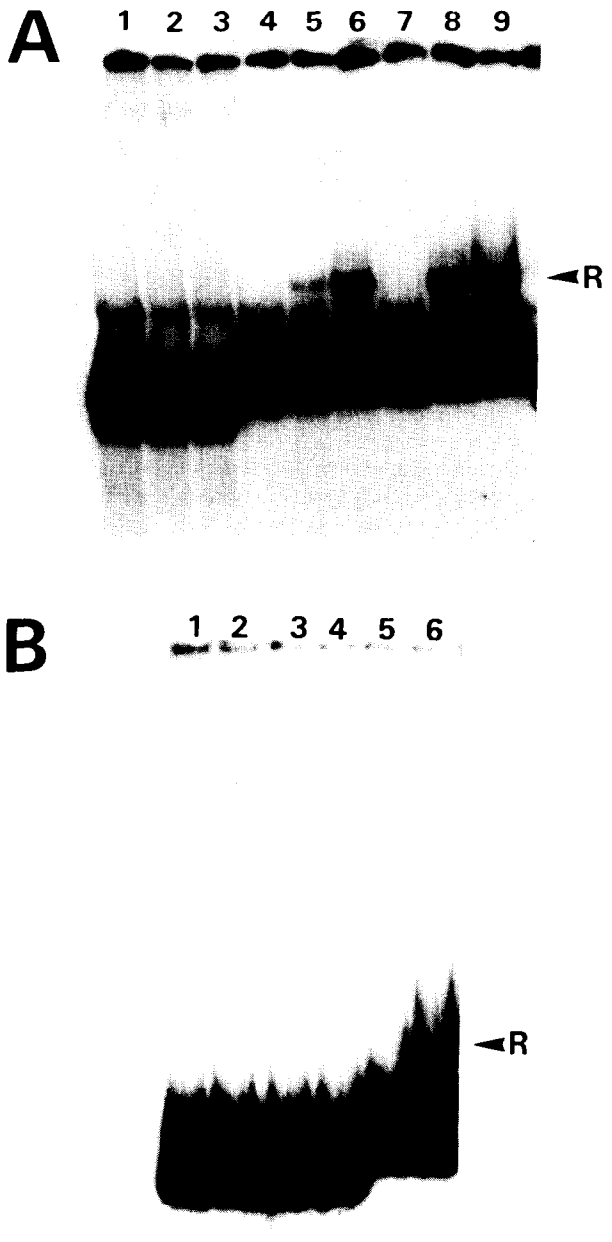

Fig. 3. Effect of sequences 3′ to the *virE vir box* on VirG binding (A) Binding of the VirG protein to *virE* promoter fragments with the *vir box* and 5, 19 or 34 additional nucleotides 3′ of the *vir box* at their 3′ end. The experiment was carried out using following fragments from plasmid pSG675: Lanes 1–3, *SalI–Eco*RV fragment (144 bp; *VB/5* sequence at the 3′ end); lanes 4–6, *SalI–AvaI* fragment (160 bp; *VB/19*); lanes 7–9, *SalI–RsaI* fragment (174 bp; *VB/34*). Lanes 1, 4 and 7, no protein; lanes 2, 5 and 8, 0.2 μg VirG protein; lanes 3, 6 and 9, 1.0 μg VirG protein. R, retarded band. (B) Binding of the VirG protein to fragments from the *lac* promoter and *vir box lac* promoter constructs. Lanes 1 and 2, *Pvu*II–*Bam*HI fragment from pSG673 (250 bp; wild-type *lacp*); lanes 3 and 4, *Pvu*II–*Bam*HI fragment from pRS3220 (262 bp; *VB:lacp*); lanes 5 and 6, *SalI* fragment from pRS9902 (350 bp; *VB/19:lacp*). Lanes 1, 3 and 5, no protein; lanes 2, 4 and 6, 1.0 μg VirG protein. R, retarded band.

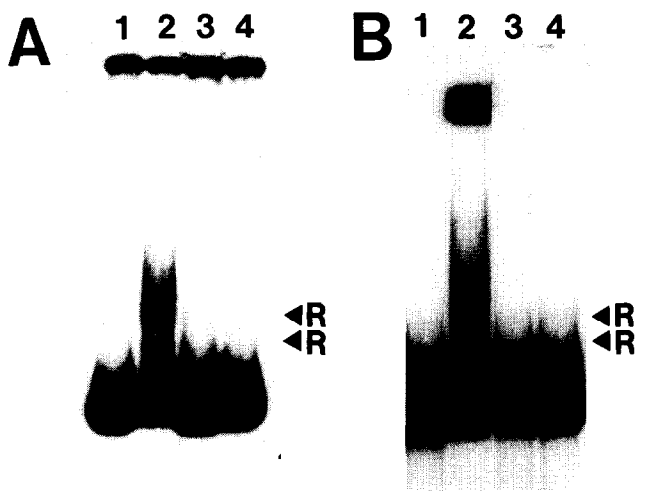

Fig. 4. Effect of 3′ and 5′ sequences on VirG binding. (A) Binding of the VirG protein to the wild type *virE* promoter and to mutated *virE* promoters. Lanes 1 and 2, *SalI–AvaII* fragment from plasmid pSG675 (250 bp, wild-type promoter); lane 3, *SalI–AvaII* fragment from plasmid pRS2013 (238 bp, deleted *vir box*); lane 4, *SalI–AvaII* fragment from plasmid pRS3324 (250 bp, *vir box* inverted). Lane 1, no protein; lanes 2–4, 1.0 μg VirG protein. R, retarded band. (B) Binding of the VirG protein to the wild-type *virA* promoter and to a mutated *virA* promoter. Lanes 1 and 2, *XmnI–XhoI* fragment from plasmid pSW169 (228 bp, wild-type promoter); lanes 3 and 4, *XmnI–XhoI* fragment from plasmid pRS3407 (215 bp, 13 bp downstream of the *vir box* deleted). Lanes 1 and 3, no protein; lanes 2 and 4, 1.0 μg VirG protein. R, retarded band.

virE promoter where the orientation of the *vir box* has been inverted at the wild type position. The gel retardation experiment shown in Fig. 4A reveals that VirG was unable to bind to fragments with this construct (lane 4) indicating that the *vir box* with 3′ and 5′ sequences different from the 3′ and 5′ wild type sequences is not recognized by VirG. In a further construct we deleted 13 bps downstream of the *virA vir box*. The finding that VirG did not bind to this construct, as shown in Fig. 4B, lane 4, provides additional proof that specific sequences 3′ of the *vir box* are necessary for VirG binding.

3.2. *The VirG binding site is sufficient to activate the* lac *promoter in response to plant signal molecules in* A. tumefaciens

We further analyzed whether the identified VirG binding site (*VBI19* sequence) could function as a cis-acting regulatory sequence of a heterologous promoter. To address this question we tested whether the VirG binding site was sufficient to activate the *E. coli lac* promoter in response to the plant signal molecule acetosyringone. The *lac* promoter is constitutively expressed in *A. tumefaciens* and the level of *lacZ* expression is affected neither by IPTG (not shown) nor by acetosyringone (Fig. 6, column no. 1).

In addition to the *lac* promoter constructs described above (*VB:lacp*, *VBI19:lacp*) we used an additional control construct where a fragment with the *VBI5* sequence at the 3′ end, which is not sufficient for VirG binding, was inserted into the *lac* promoter. As described in section 2 the different promoter constructs were subcloned into broad host range plasmids and fused with the *lacZ* coding regions. Expression of the *lacZ* gene under control of the modified *lac* promoters with and without acetosyringone was measured in *A. tumefaciens* strain A348. Fig. 5 shows that the presence of the *vir* gene fragments in the *lac* promoter decreased the constitutive expression of the *lacZ* gene in *Agrobacterium*, indicating that the corresponding upstream region is involved in transcription from the *lac* promoter in *Agrobacterium*. A similar interference of *lacZ* transcription by the presence of the *vir box* sequence has been reported by Powell and Kado [27]. The experiment shown in Fig. 5, column 4, shows that the presence of the VirG binding site (*VBI19* sequence) in the *lac* promoter resulted in a 2.5-fold induction of the *lacZ* gene in response to acetosyringone (Fig. 6, no. 4). Although the stimulation of *lacZ* expression in the presence of the plant signal molecule was small, it was highly reproducible (Standard deviation 0.124). Control experiments demonstrated that expression of the *lac* promoter with only the *vir box* sequence (pRS8501) or the *VBI5* sequence (pRR2001), neither of which are substrates for VirG binding, was not stimulated by acetosyringone (Fig. 6, columns no. 2 and 3).

Induction of the *vir* genes is mediated by the VirA/VirG two-component regulatory system. To test whether the induction of the *lac* promoter with the VirG binding site (*VBI19* sequence) by acetosyringone was dependant on both a functional *virA* and *virG* gene, the induction of the modified *lac* promoter was also assayed in *A. tumefaciens* strains with mutations in the *virA* gene (strain A 1030) and *virG* gene (strain Mx321) respec-

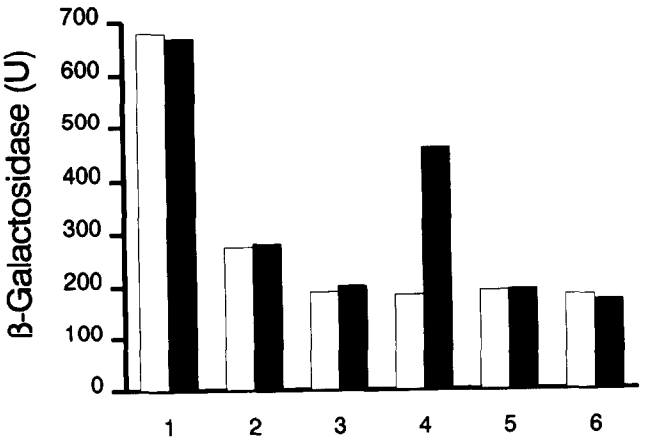

Fig. 5. Expression of the *lacZ* gene under control of the wildtype *lac* promoter and *vir box lac* promoter constructs in *Agrobacterium* strain A348 (wild-type Ti plasmid), A1030 (*virA* mutant), and Mx321 (*virG* mutant). No. 1, A348(pRS8501, *lacp*); 2, A348(pRS8401, *VB:lacp*); 3, A348(pRR2001, *VBI5:lacp*); 4, A348(pRS102.01, *VBI19:lacp*); 5, A1030(pRS102.01); 6, Mx321(pRS102.01). □, incubation without acetosyringone ■, incubation in the presence of acetosyringone.

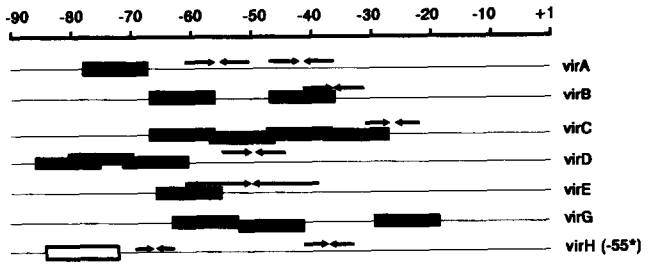

Fig. 6. Schematic representation of the promoter region of the *vir* genes. The *vir boxes* are indicates by boxes (position according to [10]) and the *vir boxes* required for full transcriptional activation according to [13] are hatched. The palindromic sequences are indicated by arrows and the transcriptional start site is labeled as +1 according to [28]. The biological activity of the *virH vir box* has not yet been determined. *The transcriptional start site of the *virH* gene [30] has not been mapped. The number indicate the position of the 3′ nucleotide of the sequence shown with respect to the translational start site.

tively. Fig. 6, columns no. 5 and 6 shows that the modified *lac* promoter on plasmid pRS10201 could not be induced by acetosyringone in the two mutated strains. This indicates that the induction of the modified *lac* promoter by acetosyringone in the wildtype strain was mediated by the VirA/VirG two component regulatory system.

4. Discussion

Although previous data strongly suggested that the *vir box* is the VirG binding site [12,15] the present study reveals that the *vir box* is not sufficient for VirG protein binding and transcriptional activation in response to plant signal molecules.

DNA binding assays using DNA fragments with increasing nucleotide length 3′ to the *vir box* demonstrate that the VirG binding site in the *virE* promoter comprises both the *vir box* as well as additional specific sequences which are contained within a 19 bp sequence 3′ to the *vir box*. Gel retardation control experiments with the *vir box* and *virE* promoter fragments subcloned into the *lac* promoter rule out the possibility that VirG binding requires just the presence of any sequence 3′ of the *vir box*. The *vir box* flanked by *lac* promoter sequences was not recognized by VirG. The finding that VirG requires specific sequences downstream of the *vir box* is supported by the finding that deletion of sequences 3′ of the first *virB vir box* resulted in a loss of inducibility of the *virB* gene [11] and the observation that the VirG protein did not bind to the DNA fragments when sequences (13 bps) 3′ to the *virA vir box* had been deleted or when the *virE vir box* was inverted.

It is not certain where the specificity lies in the 3′ region. However, the region downstream of the *virE vir box* contains a sequence of almost perfect dyad symmetry (22 out of 23 bp) which resides within the *VB/19* sequence (Fig. 1). Sequence analysis reveals that all other *vir* gene promoters, except for *virG*, also contain palindromic sequences downstream of at least one of the *vir box* sequences (Fig. 6) suggesting their possible role in VirG binding. Pazour and Das [13] have found that in *vir* gene promoters with several *vir boxes* only one of the *vir boxes* is essential for expression of the corresponding *vir* gene. Fig. 6 shows that the identified palindromic sequences are always located only downstream of the one *vir box* which is required for full transcriptional activation supporting our model that these sequences are involved in vir gene expression. Potentially the downstream sequences stabilize VirG binding and/or the lack of conserved sequences allows different binding affinities of VirG for the various *vir* boxes. Possible explanations of why the *virG* promoter does not have a palindromic sequence may be its unique dual promoter structure [14] and a different mechanism of transcriptional activation [13]. Since the palindromic sequences in the *vir* gene promoters do not share sequence homology the presence of a specific secondary structure in addition to the conserved *vir box* sequence may be necessary for VirG binding.

Since DNA binding studies of transcriptional activators of other two component regulatory systems always involved large restriction fragments with the identified conserved sequences, it remains to be determined whether these putative binding sites are sufficient for binding of the regulatory proteins. This would be especially interesting in the case of the putative OmpR binding site [28], which shares a continuous stretch of 8 out of 12 bp with the *vir box* [12]. Alternatively, it may be possible that a unique type of binding site for VirG has been developed in *A. tumefaciens vir* genes to rule out nonspecific induction by other two component regulatory systems (crosstalk). The reasons why the system is so stringently controlled could be the energy required for the expression of the large number of genes (>20) in the *vir* regulon. To our knowledge this is the first report which shows that binding of a transcriptional activator of a two component regulatory system requires both conserved and specific nonconserved sequences. Binding of a transcriptional activator protein to specific sequences with no apparent sequence homology has also been reported for the OxyR protein [29].

Our results show that the identified binding site for the VirG protein is sufficient to regulate a heterologous promoter via the VirA/VirG signal transduction chain. The expression from the *E. coli lac* promoter, which is constitutively expressed in *A. tumefaciens*, could be stimulated 2.5-fold in response to the plant signal molecule acetosyringone. The low level of induction of the *vir box lac* promoter fusion compared to the wildtype *vir* genes (9- to 113-fold [15]) may be explained as follows. First, the position of the *vir box(es)* is variable in the different *vir* genes. Although the VirG binding site was introduced in

the same relative position as in the *virE* gene, this may not be appropriate for the *lac* promoter. Second, the identified VirG binding site may not be sufficient for full induction and the specific structure of the *vir* gene promoter region is also important. A comparison of the sequence of the *lac* and *vir* gen promoters reveal that only the −10 region, but not the −35 region, of the *vir* genes share a consensus sequence which is homologous to the corresponding sequence of *E. coli* promoters [30]. The variable −35 region may play a role in the induction of these genes. In experiments similar to the ones reported here the binding site for the NtrC protein was introduced into two heterologous promoters [31]. The NtrC-phosphate was only able to activate transcription of a promoter with a binding site for the alternative sigma factor NtrA (nifHp) but not from the *lac* promoter. The difference between the results of VirG and NtrC using the *lac* promoter indicates that transcription of *A. tumefaciens vir* genes is independent of an alternative sigma factor. This conclusion is supported by the finding that the VirG protein lacks the conserved sequences shared by transcriptional activators of NtrA dependent operons.

Additional studies will be necessary to compare the properties of the VirG binding site with other bacterial cis acting regulatory sequences and elucidate the molecular mechanism of transcriptional activation using a homologous in vitro transcription system.

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