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REVIEW

Transcriptional Control of the *Agrobacterium* Virulence Genes by Two Proteins VirA and VirG

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1. Introduction

The hairy-root-inducing plasmids (pRi) and crown-gall-inducing plasmids (pTi) confer tumorigenic symptoms at wound sites on a wide variety of dicotyledonous plants upon infection by their host bacteria, *Agrobacterium rhizogenes* and *A. tumefaciens*, respectively.[1-5] These plasmids are stably maintained at a copy number of 1 to 2 in a bacterium with similar replication characteristics although pRi and pTi belong to different incompatibility groups.[6-11] Tumorigenesis by either plasmid is caused by the transfer of a defined DNA segment (T-DNA) from the plasmid into the plant nuclear genome and the subsequent constitutive production of plant phytohormones directed by the T-DNA.[12-19] The T-DNA also carries genes coding for enzymes that synthesize the unique amino acid derivatives called opines (e.g. agropine, agrocinopine, nopaline, and octopine),[20,21] by which pRi and pTi have expediently been classified. The 25-base-pair (bp) imperfect direct repeats at both extremities of T-DNA are indispensable in *cis* for the T-DNA transfer, but never are the other portions inside the T-DNA.[22-24] Plasmid genes essential for the T-DNA transfer are located in the virulence (*vir*) loci outside the T-DNA.[22,25-27] Besides, several crucial genes (*chv*, chromosomal virulence genes) are scattered on the *Agrobacterium* chromosome.[28] The plasmid *vir* genes (about 20 genes) constitute five or more transcriptional units (*virA* to *virG*).[29-31] Their expression is tightly regulated as a regulon, being inducible by plant phenolic compounds such as acetosyringone.[29,32-34] Two member genes of the virulence regulon, *virA* and *virG*, are critical for inducible expression of the whole *vir* genes.[35] Here we will summarize organization and expression modes of the *vir* genes, and then delineate mechanisms of transcriptional regulation of the *vir* genes by the *virA* and *virG* gene products (VirA and VirG). Experimental results introduced below are those mainly done with the agropine-type pRiA4, although those with the octopine-type pTiA6, the nopaline-type pTiC58, and other plasmids are also occasionally presented for comparison. The *Agrobacterium* VirA-VirG system belongs to the so-called two-component regulatory system that consists of the sensor component and the regulator compo-

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nent.[36,37] It has been known that various two-component regulatory systems control expression of a variety of sets of bacterial genes upon environmental variations. Also discussed is an example of cross-talk during signal transduction between different two-component regulatory systems.

2. Organization of the pRiA4 *vir* genes

Using transposon insertion mutagenesis, many avirulent mutants of pRiA4 have been constructed. Their transposon insertion sites are localized in a region of about 26 kb. The gene organization and nucleotide sequence of the coding portions within this region (*vir*) is highly homologous to those of the pTiA6 and pTiC58 *vir* loci in which nonpathogenic derivatives of pTi carry mutations (see Figure 1).[29-31,38-44] All *vir* genes except *virC* are transcribed

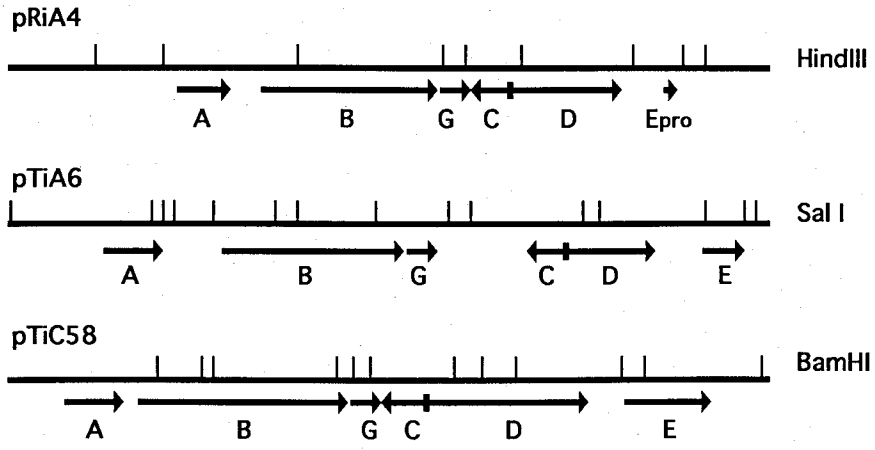

Figure 1. Transcription units of the *vir* loci of three plasmids, pRiA4, pTiA6, and pTiC58. Each arrow indicate the size and direction of a transcription unit. Restriction map of pRiA4, pTiA6, and pTiC58 are those of refs. 30, 29, and 31, respectively.

in the same direction. The *virA* and *virG* loci contain a single gene, while the *virB*, *virC*, and *virD* loci are composed of 11, 2 and 4 genes, respectively. The assignments of these *vir* genes from particular open reading frames on the DNA sequences are based on (i) genetic complementation of transposon insertion mutants; (ii) alignments of DNA sequences between *vir* loci of various plasmids; and (iii) detection of predicted gene products. In each case an ATG codon is present at a site plausible for translation initiation. However, translation of *virG* is initiated at an unusual TTG codon but not conventional ATG and GTG codons.[39] Although base substitution from TTG to ATG does not seem to affect inducible expression of *vir*,[45] this

TTG start codon is conserved in all *virG* genes so far sequenced. This may contribute to delicate control of the *virG* translation efficiency (e.g. rather moderate translation initiation) in conjunction with the complex promoter organization for *virG* as described below.

Obvious structural differences between pRiA4 and pTi are: (i) the *virE* locus is missing in pRiA4 though its promoter region is retained; (ii) the latter half of the second gene of the pRiA4 *virD* operon (*virD2*) differs from that of pTiA6 but is similar to that of pTiC58; and (iii) the pRiA4 *virD3* gene thoroughly differs from the corresponding gene of pTiA6. Phylogenic distances between the plasmids deduced from the resemblance of each *vir* gene are constant: for instance, every *vir* gene of pRiA4 is more similar to that of pTiC58 than that of pTiA6. Therefore, it is evident that all of the *vir* genes have evolved, as a set, from the respective common ancestor genes without shuffling within the *vir* region. In contrast to the close similarity of the coding regions, the spacer regions between the *vir* operons and also between the *vir* genes are generally less conserved, and some spacer regions involve IS-like sequences: the spacers between *virG* and *virC* of pTiA6 and between *virA* and *virB* of pRiA4 contain an IS*66*[46] and a portion of IS*66*-like sequence,[41,47] respectively. Thus, DNA rearrangements resulting from IS transpositions seem to have occurred during evolution of the *vir* gene sets. All mutations within the pRiA4 *vir* genes, except for *virD3*, either eliminate (*virA*, *virB*, *virD*, and *virG*) or restrict (*virC*) the ability of *Agrobacterium* to develop tumorigenic symptoms on plants.[29,30] Mutations in the pTi *virE* gene cause attenuated pathogenicity as *virC* mutations.[48] Nevertheless, pRiA4 naturally lacking the *virE* gene is efficiently pathogenic, and the supplement of the pTi *virE*$^+$ gene to pRiA4 does not enhance pathogenicity.[40] The pRiA4 genome might contain an unidentified functional homolog of *virE*. It is unknown why the non-essential *virD3* gene is located within the *vir* loci. A progenitor gene of *virD3* may have been indispensable for pathogenicity on some plant species, or may have been inserted into the *vir* loci together with the latter half of *virD2* during evolution.

3. Inducible expression of the *vir* genes by plant factors

Transcription from the six *vir* loci of pRiA4 under the presence or absence of a plant factor, acetosyringone, (inducing or noninducing conditions, respectively) has been analyzed by the S1-mapping procedure.[33] The results indicate that transcription of *virB*, *virC*, *virD*, and *virE* occurs only under the inducing conditions and that *virA* and *virG* are transcribed at low levels under the noninducing conditions but higher levels under the inducing conditions. Essentially the same expression patterns have been shown with pTi by analysis of translation products of the *vir* genes.[29,32] Induction of *vir* expression occurs in plant culture medium (pH 5.5) but not in bacterial broth medium (pH 7.2), the acidic conditions being requisite.[49] Inducible mRNA synthesis from *virB*, *virC*, *virD* and *virE* is initiated from the respective unique sites. By contrast, transcription from *virA* starts at two sites separated by 141 bp, each directed by different promoters, the upstream and downstream promoters; and the *virG* gene is preceded by three tandem promoters (upstream, middle, and downstream promoters) at intervals of 21 bp and 27 bp. These *virA*/*virG* promoters are properly used. The upstream promoter of *virA* operates at a constantly low level regardless of the conditions used, while the downstream promoter of *virA* works at a comparable low level under the noninducing con-

ditions and at a much increased level under the inducing conditions. The middle promoter of *virG* has an operation mode similar to the upstream promoter of *virA*. The upstream and downstream promoters of *virG* function only under the inducing conditions, and the former contributes to the majority of inducible mRNA synthesis from *virG* (more than 80%).

Mutations in either gene of *virA* and *virG* abolish inducible expression of every *vir* gene, but none of the other *vir* mutants affect *vir* induction. These facts mean that the *vir* loci constitute a regulon whose expression is positively controlled under a regulatory system composed of VirA and VirG.

4. Vir box sequences present in the *vir* promoter regions

Generally, the -35 and -10 regions of *Escherichia coli* promoters have the respective consensus sequences (TTGACA and TATATT, respectively), and their spacer lengths are kept at about 17 bp (see Figure 2b).[50,51] *E. coli* promoters introduced into *Agrobacterium* cells are

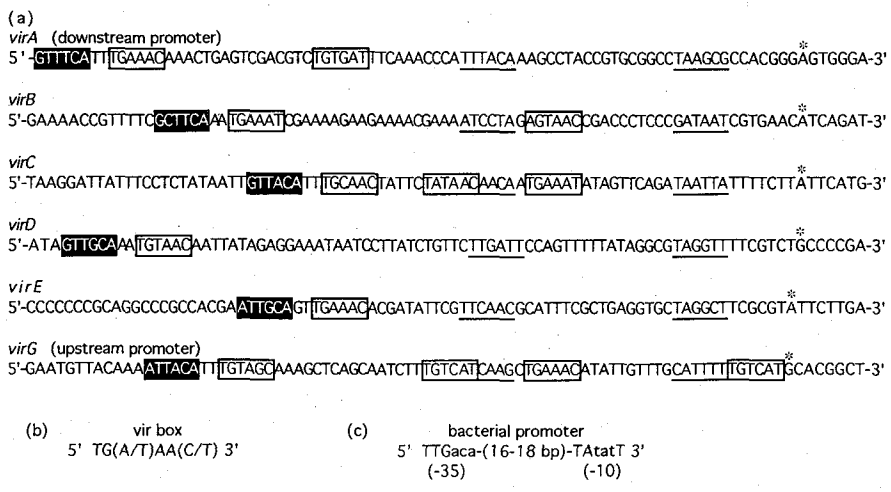

Figure 2. Nucleotide sequence of the *vir* promoter regions.[33] (a) shows DNA regions upstream of inducible mRNA start sites. An asterisk points the transcription initiation site, and underlines indicate the -35 and -10 regions of promoter. Vir box sequences and inverted vir box sequences are marked by open and shading boxes, respectively. (b) and (c) represent the consensus sequence of vir boxes and bacterial promoters, respectively. Upper-case letters show the three most highly conserved bases in each region.

capable of synthesizing RNA from a site identical to that in *E. coli*, implying structural similarity between *Agrobacterium* and *E. coli* promoters.[52] Actually, the consensus structure deduced from *E. coli* is well conserved in the *vir* constitutive promoters (the *virA* upstream promoter and the *virG* middle promoter), while less so in the *vir* inducible promoters, particularly at the -35 region (Figure 2a). These facts suggest that RNA polymerase by itself is

unable to interact with the *vir* inducible promoters. The *virD* promoter, which is inactive unless a plant factor exists, exceptionally carries the well-conserved promoter sequences. This discrepancy appears to associate with presumable repression under the noninducing conditions of the pRiA4 *virD* gene by the chromosomal *ros* gene product, as reported with pTiC58.[53]

Since elimination of the DNA region (about 70 bp) upstream from the mRNA start site of *vir* abolishes inducible transcription by a plant factor,[54] DNA signals required for induction should be present in the promoter regions. There seems to be no obvious extended sequence similarity at given distances from the RNA start sites. However, characteristic hexamer blocks with the consensus sequence of 5'-TG(A/T)AA(C/T)-3' (vir box) appear frequently: one to four *vir* boxes for each *vir* gene (Figure 2a).[33,55] These vir boxes are helically phased though do not always emerge at consecutive helical turns, and their phase is nearly opposite to that of the -35 and -10 regions of the promoter. Besides, the most upstream vir box is invariably preceded by an additional vir box in the inverted orientation. These facts strongly support the view that the vir boxes are *cis*-acting elements for recognition by a transcription factor.

5. Structure and function of VirG: Interaction of VirG with vir box sequences

VirG is composed of 241 amino acid residues, and its N-terminal half resembles the corresponding portions of various two-component regulatory system's regulatory components.[31,39,44] By contrast, its C-terminal half is generally less similar to the other regulatory components but is close to some regulatory components, intimating that the regulatory components can be classified into subgroups.[36,37] On the basis of such similarity and characteristics of *virG* mutants, VirG has been thought to be a transcriptional activator that directly binds to the regulatory regions for each *vir* gene. The hexamer vir boxes offered in the preceding section have been candidates for recognition signals by VirG. In fact, transcriptional activation occurs with synthetic promoters including phased vir box sequences.[56] In addition, VirG purified from overproducing *E. coli* cells binds to the *vir* promoter regions including the vir boxes.[45,57] Detailed analyses by methylation inhibition footprinting experiments (see Figure 3)[45] indicate that (i) every vir box including the inverted one interacts with VirG from the major groove of DNA; (ii) the initial interaction between VirG and DNA occurs at or near the inverted vir boxes, probably by two VirG molecules; (iii) additional VirG molecules then bind to the downstream DNA region cooperatively with the preceding VirG molecules in a head-to-tail manner (binding-cade model); and (iv) the interaction of VirG with the vir box sequence seems to be attended with alteration of the DNA tertiary structure. Since the major grooves of the vir boxes array at an interval of integral multiples of 11 bp, VirG molecules should line up in tandem, closely to one another, along one side of the DNA helix. The cooperative binding of VirG probably guides RNA polymerase to the promoter by specific interaction of VirG with the enzyme, by change in the conformation of the promoter region, or by a combination of these two actions. As VirG molecules bind the DNA helix from one side that is nearly opposite to the polymerase binding site, the DNA region, thought to interact with RNA polymerase, is free from the contact of VirG as illustrated in Figure 4. A truncated C-terminal half of VirG exerts similar DNA binding activity.[58] The DNA binding activity of

VirG described here is all exerted by protein preparations derived from *E. coli* without plant signals.

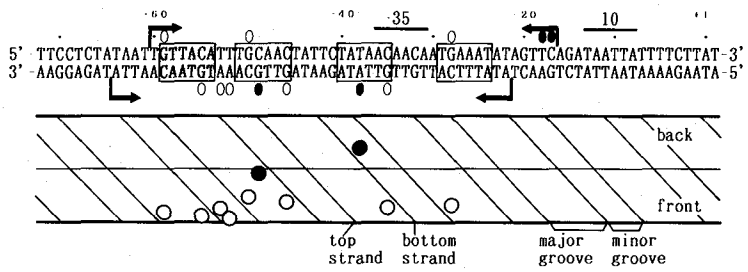

Figure 3. Summary of footprinting experiments with DNase I protection and methylation inhibition upon VirG binding to the *virC* promoter region.[45] DNA sequence of the upstream region of *virC* accompanied by its helix map (10.5 bp per turn) is shown. Boundaries of the DNA regions protected from DNase I digestion are shown by arrows. The residues where methylation is inhibited or enhanced are marked by an open circle or a filled circle, respectively. The transcriptional start site is indicated by +1, from which the upstream residues are numbered with the minus sign. The -35 and -10 regions of the *virC* promoter are indicated by -35 and -10, respectively. The phased vir box sequences are boxed, and inverted one with shading.

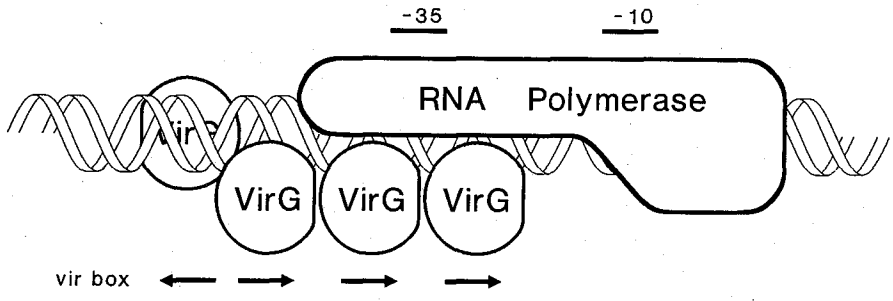

Figure 4. Model for the interaction among VirG, RNA polymerase, and DNA.[45] Two VirG molecules are first bound to the inverted vir boxes, and then additional molecules are cooperatively combined in a head-to-tail manner with DNA at the consecutive major grooves along one side of DNA helix. At the major groove lacking the vir box, DNA may loosely interact with VirG, or bend for leading to cooperative binding with VirG skipping at that major groove as discussed previously.[45,69] This cooperative binding allows RNA polymerase to interact with the promoter from the other side of the DNA. To distinguish between a head and a tail, VirG is depicted as a cut sphere. Arrows indicate the position and direction of the vir box sequences, and two upper bars show the -35 and -10 regions of the promoter.

6. Structure and function of VirA

6.1 Structure of VirA

For plant factors to signal *vir* expression, extracellular recognition is required. This process is mediated either directly or indirectly by VirA, which is composed of 829 amino acid residues.[41,42] As schematically shown at the top of Figure 5, VirA consists of three

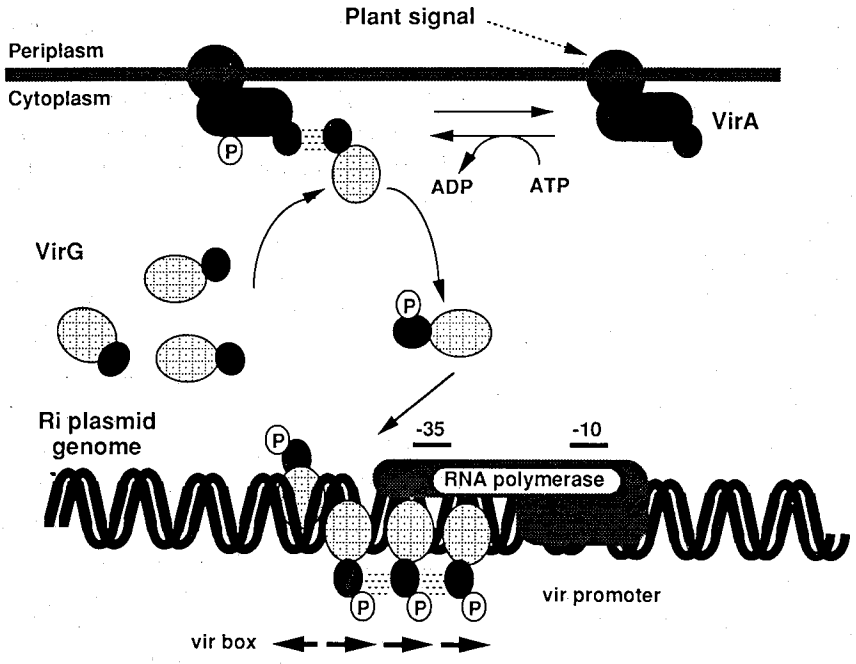

Figure 5. Schematic illustration of signal transfer from VirA to VirG followed by transcriptional activation. VirA and VirG are respectively composed of three and two domains, among which the C-terminal VGL domain of VirA and the N-terminal signal receiver domain of VirG share their structures. The circled letter P indicates the phosphoryl group on His-474 of VirA or Asp-52 of VirG, and the set of five thin broken lines symbolize molecular association between VirA and VirG or between two VirG's. The ternary complex is the same as in Figure 4.

domains. Its N-terminal half contains two membrane-spanning regions separated by about 220 residues, and their inside and outside regions are periplasmic and cytoplasmic, respectively.[59,60] Such membrane topology is frequently seen in the sensor component proteins. The VirA periplasmic domain has therefore been expected to directly sense plant signals. However, this domain can be totally deleted from VirA without loss of function, while the deletion together with the second transmembrane segment severely diminishes the response to plant factors.[59] Moreover, two bacterial proteins (p10 and p21) but not VirA have recently

been found to bind an acetosyringone analog, α-bromoacetosyringone,[61] suggesting that the primary sensor is not VirA. Thus, the actual sensor is still equivocal, but it is certain VirA is located on the signalling line in between sensing of plant factors and induction of *vir* expression. The C-terminal half of VirA is extremely close to those of other two-component regulatory system's sensor components.[36,37] Therefore, this portion should conduct function(s) common to all sensor components, such as signal transfer from sensors to regulators. In accord with this argument, mutations within this region commonly influence inducible expression of *vir*. Unlike other sensor components, however, VirA has an additional domain at the most C-terminal end.[37,41,42,62,63] This domain consists of about 115 amino acid residues, and is called the VirG-like domain (VGL domain) because of its resemblance to the N-terminal half of VirG. Thus it is likely that gene duplication has occurred during evolution of the *virA* and *virG* genes. VGL domain mutants show poorly inducible expression of the *vir* genes by a plant factor, and generate no or reduced tumorigenic symptoms on plants,[64] the possible mechanism for which will be presented below.

6.2 Autophosphorylation of VirA

For biochemical analysis of VirA, attempts have been made for overproduction of the intact VirA protein in *E. coli*. However, the N-terminal half of VirA is inhibitory for overproduction, and only N-truncated versions of VirA (VirA') have been overproduced and characterized *in vitro*. These VirA' derivatives are capable of phosphorylating themselves in the presence of ATP, if their deletions do not extend beyond His-474.[64-66] Furthermore, VirA' versions with larger N-truncations have generally higher autophosphorylating activities.[64] The His-474 residue is a target of autophosphorylation.[65] Substitution of this residue by Gln abolishes autophosphorylation function, the ability to induce *vir* expression, and pathogenicity on plants, indicating autophosphorylation as being essential in signal transduction for transcriptional activation.[65] Deletions of the VGL domain from VirA' cause almost no effect on the ability of autophosphorylation.[64] Based on these facts and other several lines of circumstantial evidence, we believe that the active center of VirA kinase is usually masked by its N-terminal half, and upon recognition of plant factors, become unmasked for activation.

6.3 Phosphotransfer from VirA to VirG

When VirG is mixed with VirA' in the presence of ATP *in vitro*, VirG together with VirA' is phosphorylated. The phosphate of phospho-VirG comes from phospho-VirA' but not ATP because VirG is similarly phosphorylated by the purified phospho-VirA', concurrently with dephosphorylation of phospho-VirA'.[67] Asp-52 within the N-terminal half of VirG is the phosphorylation target. Thus, the N-terminal half of VirG is a signal receiver domain. Site-directed mutagenesis toward Asp-52 and neighboring residues of VirG leads to an extreme reduction of pathogenicity.[58] These facts support the view that phosphotransfer is an important process involved in signal transduction required for tumorigenesis.

The most C-terminal portion of the VirA cytoplasmic segment resembles the VirG signal receiver domain as described above; particularly three residues (Asp-9, Asp-52, and Lys-102) critical for acquiring the phosphate-group[37] are completely conserved.[41] This VGL domain does not contribute to the enzymatic activity of autophosphorylation but enhances in-

teraction between VirA and VirG molecules since VGL deletion mutants slightly decrease the efficiency of phosphotransfer reaction *in vitro*.[64] Therefore, the VirA-VirG interaction appears to occur through two homologous regions, namely the VGL domain of VirA and the N-terminal signal receiver domain of VirG, mimicking the oligomerization process of VirG molecules. Although this modest decrease in phosphotransfer activity *in vitro* does not seem to account for the drastic phenotype alteration *in vivo* (reduced or no pathogenicity of VGL mutants), this contradiction is probably derived from the difference of VirA molecules tested *in vivo* and *in vitro* : the former VirA is a native membrane-anchored protein (VirA) as seen in Figure 5, while the latter VirA is an N-truncated soluble protein (VirA'). Therefore, VirA-VirG interaction is likely to be more restricted than VirA'-VirG interaction, and the peculiarity of interaction enhancement is thought to more elevate the opportunity of contact between VirA and VirG than between VirA' and VirG.

7. VirG-dependent transcription *in vitro*

Transcriptional activation by VirG of the *vir* genes has been tested *in vitro*. RNA polymerase holoenzyme purified from *Agrobacterium* has characteristics similar to those of *E. coli* (e.g. requirement of -35 and -10 sequences), although the molecular size of sigma factor differs with each other.[68] This enzyme alone is unable to transcribe the inducible *virC* and *virE* genes, as expected, but the addition of VirG purified from overproducing *E. coli* cells allows the enzyme to synthesize mRNAs, the start sites of which are the same as those made *in vivo*. Under moderate acidic conditions (pH 6.5-7.0), coupling with phosphotransfer reaction by VirA' to VirG gives more efficient mRNA synthesis, but under neutral conditions (pH 7.3-7.7), no such activation, or rather reduction of activity, has been observed by phosphorylation of VirG.[52] Therefore, it is obvious that VirG has enough potential for transcriptional activation without being phosphorylated at least under the conditions used *in vitro*, and that phospho-VirG promotes transcriptional activation more efficiently than nonphospho-VirG in the rather acidic conditions where *in vivo* expression of *vir* is induced by plant factors. Since phosphotransfer from VirA to VirG appears critical for *vir* expression *in vivo*, functional nonphospho-VirG *in vitro* seems to be interpreted as that VirG phosphorylation is essential for transcriptional activation only when the concentration of VirG is low, and function of phospho-VirG can be compensated by an excess of nonphospho-VirG. In other words, transcriptional activation by VirG is determined by both concentration and quality of VirG molecules. VirG operates as a positive transcription factor but not as an alternative sigma factor because the RNA polymerase core enzyme is unable to substitute the holoenzyme in the *in vitro* transcription system.[52]

8. Cross-talk between the virulence and phosphate regulons

Many kinds of regulatory systems for bacterial regulons belong to the two-component regulatory system composed of the sensor and regulators (e.g., VirA-VirG, PhoR-PhoB, EnvZ-OmpR, NtrB-NtrC, CpxA-SfrA, and DctB-DctD). Corresponding components of diffe-

rent systems contain conserved domains. About 200 amino acids of the C-terminal region (autophosphorylation domain) are conserved among the sensor components. In the regulatory components, about 120 amino acids of the N-terminal region (phosphate receiver domain) are conserved.[36,37] In addition, the regulatory components can be subdivided into several groups based on the similarity of their C-terminal regions as noted above. Among the subclasses, VirG of the virulence regulon, PhoB of the phosphate regulon, and OmpR of the osmolarity regulon, display a high degree of similarity along their entire amino acid sequences. Genes activated by PhoB and OmpR are preceded by *cis*-acting elements containing hexamer blocks in a helical-phase specific manner, similar to the vir box sequence.[69] Therefore, these three regulatory components are likely to have common mechanisms for both signal transduction and transcriptional regulation, allowing us to suppose that cross-talk among the three regulons may occur during responses to environmental stimuli. Indeed the pRiA4 and pTiA6 *virG* genes are expressed inducibly not only by plant phenolics but also by starvation for inorganic phosphate in both *Agrobacterium* and *E. coli* cells.[70,71] This response of *virG* to phosphate limitation *in vivo* does not require VirG, but depends entirely on the presence of PhoB. The purified PhoB protein binds to the vir boxes upstream of the *virG* gene as if it were VirG.[71] It has been thus concluded that cross-talk between the two regulons occurs during the recognition of a DNA signal by the regulatory protein, confirming that transcriptional regulation by two-component regulatory systems, at least by the VirA-VirG and PhoR-PhoB systems, is mediated through a common mechanism. There being such cross-talk is compatible with the view that the DNA signals for VirG and PhoB have evolved from a common ancestral *cis*-acting element and have diverged into a stage at which cross-recognition can barely occur. Simultaneously, two-component regulatory systems by which signals are transferred onto these DNA elements upon environmental stimuli are thought to have evolved from a common ancestral system. During evolution, shufflings of functional domains among their component proteins may have happened, creating a variety of systems. Nevertheless, the mechanisms themselves for transcriptional activation have most likely been conserved, since cross-talk as described here can occur over species.

A DNA signal frequently works as the *cis*-acting element for two or more different transcription factors in eukaryotic cells,[72] but this is the first case in prokaryotic cells of the recognition of a DNA signal by two positive regulators, resulting in cross-talk between two regulons. Prokaryotic *cis*- and *trans*-factors for transcriptional activation also might be linked to one another in some complicated way and form networks of regulatory systems, as in eukaryotes.

9. Epilog

In this issue, we have delineated mechanisms for transcriptional activation of the *Agrobacterium vir* genes triggered by plant factors, as illustrated in Figure 5. This response is mediated by the sensor protein VirA and the transcription factor VirG, and phosphate transfer between them is the noumenon of signal transduction. The VGL domain at the C-terminus of VirA enhances molecular interaction between VirA and VirG presumably imitating dimerization of VirG, and thereby elevating signal transfer. Transcriptional activation is achieved

by conducting RNA polymerase holoenzyme to the *vir* promoter regions through cooperative binding of VirG to the phased vir box sequences upstream from each *vir* gene. All of these processes have been demonstrated by *in vitro* experiments except sensing plant phenolics by VirA. Based on the observed cross-talk between the virulence and phosphate regulons, we have discussed the possibility that the VirA-VirG regulatory system may be connected to other two-component regulatory systems, thereby forming complex regulatory networks.

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