The EMBO Journal vol.8 no.7 pp.1919-1925, 1989

Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*

Leo S.Melchers, Tonny J.G.Regensburg-Tuïnk, Robert B.Bourret[1], Norbert J.A.Sedee, Rob A.Schilperoort and Paul J.J.Hooykaas

Department of Plant Molecular Biology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands and [1]Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

Communicated by P.van de Putte

The *virA* gene of *Agrobacterium tumefaciens* encodes an inner membrane that mediates the transcriptional activation of virulence genes in response to plant signal molecules. We report here a functional analysis of the N-terminal, C-terminal and periplasmic domains of VirA in transmembrane signalling. First, we show that VirA has a transmembrane topology by analysis of the alkaline phosphatase activities, determined by several *virA-phoA* gene fusions. Second, we report here the construction of several *virA-tar* chimeric genes, in which the 3'-coding region of *virA* is conserved to study transmembrane signalling, as well as the construction of a set of *virA* deletion mutations. Results of analyses of *vir* induction behaviour and tumour inducing abilities of agrobacteria carrying these mutant genes do not support existing models for the chemoreceptor function of the VirA periplasmic domain. We demonstrate that the periplasmic domain of VirA can be either replaced by a corresponding region of the *E.coli* chemosensory protein Tar or even totally deleted from VirA without a loss of function. Here, we present a model of VirA which involves a receptor function for the second membrane-spanning domain and an intracellular signalling function for the cytoplasmic domain of VirA. In addition, we show that VirA plays a role in determining the sensitivity for pH and temperature in acetosyringone-mediated *vir* induction, and we propose a role for the VirA periplasmic domain in detection of the external pH conditions.

Key words: Escherichia coli Tar protein/signal transduction/plant-bacterial interaction/VirA transmembrane regulatory protein/virulence gene expression

Introduction

Transmembrane signalling systems in both prokaryotes and eukaryotes mediate a variety of cellular responses to environmental stimuli. The soil bacterium *Agrobacterium tumefaciens* possesses the bizarre ability to provoke tumours on dicotyledonous plants (for recent reviews see Koukolikova-Nicola *et al.*, 1987; Melchers and Hooykaas, 1987). The virulence (*vir*) genes *virA* and *virG* of the tumour inducing (Ti) plasmid of *Agrobacterium* enable the bacterium to sense specific plant signal molecules and to mediate two distinct types of responses, i.e. chemotaxis (Shaw *et al.*, 1988) and induction of *vir* gene expression (Stachel and Zambryski, 1986). The plant signal molecules involved are acetosyringone (AS) (Stachel *et al.*, 1985) and structurally related compounds, including certain lignin precursors (Spencer and Towers, 1988; Melchers *et al.*, 1989). The chemotaxis system of *A.tumefaciens* facilitates migration towards plant cells that are susceptible to transformation. Activation of the virulence system of *Agrobacterium* leads to the formation of single-stranded DNA molecules called T-strands, which are transferred to plant cells and become integrated into the plant genome as T-DNA. The expression of specific T-DNA genes results in the transformation of normal plant cells into tumour cells.

The mechanism by which AS activates the transcription of the Ti plasmid *vir* operons is unknown. The regulatory proteins VirA and VirG show similarity to a two-component sensor/regulator system that is highly conserved in a variety of prokaryotes (Ronson *et al.*, 1987). VirA is an inner membrane protein that most likely functions as a chemoreceptor to sense the presence of plant signal molecules and thereupon activates VirG (Leroux *et al.*, 1987; Melchers *et al.*, 1987). The activated form of VirG is thought to bind to the *vir* promoters, thereby stimulating *vir* gene expression (Melchers *et al.*, 1986; Winans *et al.*, 1986). Comparison of bacterial proteins with known or suspected signalling roles have revealed sequence motifs that probably act as transmitter in mediating protein-protein communication (Ronson *et al.*, 1987; Kofoid and Parkinson, 1988). Some of these proteins, CheA (Hess *et al.*, 1987), NtrB (Ninfa and Magasanik, 1986) and EnvZ (Igo and Silhavy, 1988) are known to have kinase activities. Intracellular signalling in *Agrobacterium* via VirA and VirG might therefore involve protein phosphorylation similar to the prokaryotic systems mentioned above and many eukaryotic systems.

The transmembrane receptor proteins Tar, Tsr, Trg and Tap of the *Escherichia coli* chemotaxis system are among the best characterized transmembrane proteins (for reviews see Ordal, 1985; Simon *et al.*, 1985). These signal transducers contain an extracellular (periplasmic) receptor domain that monitors the level of attractant and repellent chemicals in the environment and an intracellular (cytoplasmic) signalling domain that controls the rotational behaviour of the flagellar motors. In a recent report on Tsr, Ames and Parkinson (1988) suggest that transmembrane signalling may involve direct propagation of conformational changes between the periplasmic and cytoplasmic portions of the receptor protein.

Here we describe studies of the structure and functions of the VirA protein in virulence of *Agrobacterium*. We provide evidence that the region between the two hydrophobic segments of VirA is periplasmic, whereas the region carboxy-terminal to the second hydrophobic domain is cytoplasmic. Functional analysis of *virA* mutants, including *virA-tar* fusions and *virA* deletion mutants, shows that the

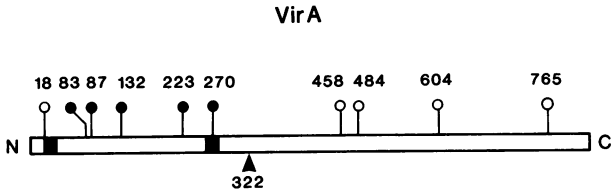

Fig. 1. Fusion joints of VirA−PhoA hybrid proteins. The two hydrophobic regions [amino acid positions 18−39 (TM1) and 260−280 (TM2)] are indicated by black boxes. The vertical line indicates the VirA−PhoA fusion joints. Closed circles (●) refer to hybrid proteins that have high alkaline phosphatase activity (18−30 U), whereas open circles (○) indicate the absence of significant AP activity (<4 U). The numbers at the top indicate the amino acid at which the fusion has occurred. The arrow refers to the fusion site of VirA−LacZ (β-galactosidase activity, 500 U).

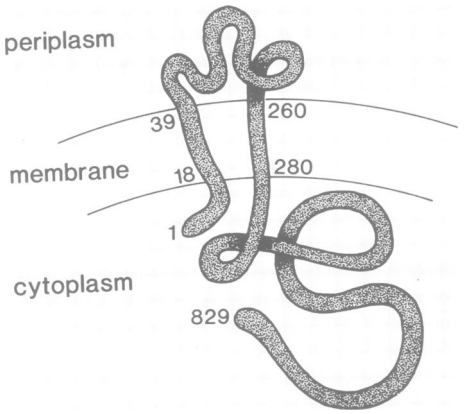

Fig. 2. Schematic view of the membrane topology of VirA.

periplasmic domain of VirA is not necessary for *Agrobacterium vir* induction. A possible sensory function for the second membrane-spanning domain of VirA in signal transduction is discussed.

Results

Membrane topology of VirA

VirA is thought to function as a signal transducer for plant phenolics and is localized in the inner membrane of *Agrobacterium* (Leroux *et al.*, 1987; Melchers *et al.*, 1987). In these reports it was proposed that VirA had a simple transmembrane topology analogous to that of the well-known *E.coli* chemoreceptors Tar and Tsr (Krikos *et al.*, 1983). In order to investigate the membrane topology of VirA, we constructed various *virA−phoA* gene fusions encoding hybrid proteins in which the catalytic domain of alkaline phosphatase (PhoA) was joined to various lengths of the N terminus of VirA. The *phoA* coding region of the fusions is intact, except that the entire 5′ region, which codes for the signal sequence of pre-PhoA, is missing (Hoffman and Wright, 1985; Manoil and Beckwith, 1986). Since alkaline phosphatase (AP) must be exported to the periplasm to acquire enzymic activity, VirA−PhoA hybrid proteins will be enzymically active only if PhoA is linked to a VirA domain that is normally located on the periplasmic side of the membrane. The different *virA−phoA* gene fusions, shown in Figure 1, were constructed by cloning *virA* DNA fragments of various lengths in frame with the *phoA* coding region of pRL751. DNA sequence analysis of the fusion sites revealed that all of the fusions were in the correct reading frame of *virA*. We found that none of these constructs conferred on *Agrobacterium* an increased alkaline phosphatase activity after introduction on an *incP*-class vector (data not shown). This is probably due to the very small amounts of VirA present in *Agrobacterium* (Melchers *et al.*, 1987). Therefore, we used a high copy number pUC-plasmid to increase the number of the VirA−PhoA hybrid proteins per cell and analysed the *PhoA* activity encoded by the constructs in *E.coli*. First we confirmed that the VirA protein expressed in *E.coli* is similar in its localization and size to the VirA product in *Agrobacterium*. Western blot analysis revealed that the VirA protein produced in *E.coli* (pRAL3255), which contains a wild-type *virA* gene in plasmid pUC19, is associated exclusively with the membrane part of *E.coli* and has the same mol. wt as the native VirA protein of *Agrobacterium*. The AP activities of the different VirA−PhoA hybrids were determined in *E.coli* strain CC118 (*phoA*$^-$) and the results are shown in Figure 1. High levels of AP activity were found for four PhoA fusions that map within the predicted periplasmic VirA domain and for one PhoA fusion present within the second membrane spanning domain of VirA. Hybrid proteins that contain PhoA linked to VirA beyond its N-terminal (amino acid position 18)—or within its C-terminal part (amino acid positions 458, 484, 604, 765) displayed no significant AP activity. Additional evidence for a cytoplasmic location of the latter region was provided by the high β-galactosidase activity found for the VirA−LacZ fusion shown in Figure 1. The results presented here provide evidence for the transmembrane topology of VirA as shown in Figure 2. The two hydrophobic segments (TM1 and TM2) that flank the periplasmic domain in the N-terminal region of VirA serve to anchor this protein in the cytoplasmic membrane. A large C-terminal region (~550 amino acids) is located on the cytoplasmic side of the membrane.

Site directed mutagenesis of VirA

In order to characterize the functions of the distinct VirA protein domains in stimulus detection and transmembrane signalling, we constructed a set of *virA−tar* hybrid genes and several *virA* mutants containing specific deletions. These constructs were introduced into strain LBA2524, which lacks the entire *virA* gene and contains a Ti plasmid provided with a *virB−lacZ* gene fusion in *cis*, to monitor *vir*-gene expression. The resulting strains were tested for the activation of *vir*-gene expression in response to AS and their ability to induce tumors on *Nicotiana glauca*.

To be able to construct the desired *virA−tar* chimeric genes, in which analogous regions of *virA* and *tar* had been exchanged, we needed suitable restriction sites in *virA* for cloning. By site-directed mutagenesis of *virA* we created unique recognition sites for the restriction enzymes *Xba*I and/or *Hpa*I within the nucleotide sequence of *virA*, see Figure 3A. These sites fall within the coding region that corresponds to the periplasmic domain of VirA and are compatible with the *Nhe*I or *Eco*RV sites of the *tar* construct. Before using these *virA*-mutants in creating the *virA−tar* constructs, we tested possible effects of the amino acid substitutions in the periplasmic domain of VirA. From each plasmid, pRAL7001 (*virA*[Hpa]), pRAL7002 (*virA*[Xba]) and pRAL7003 (*virA*[Xba;Hpa]), we tested nine constructs,

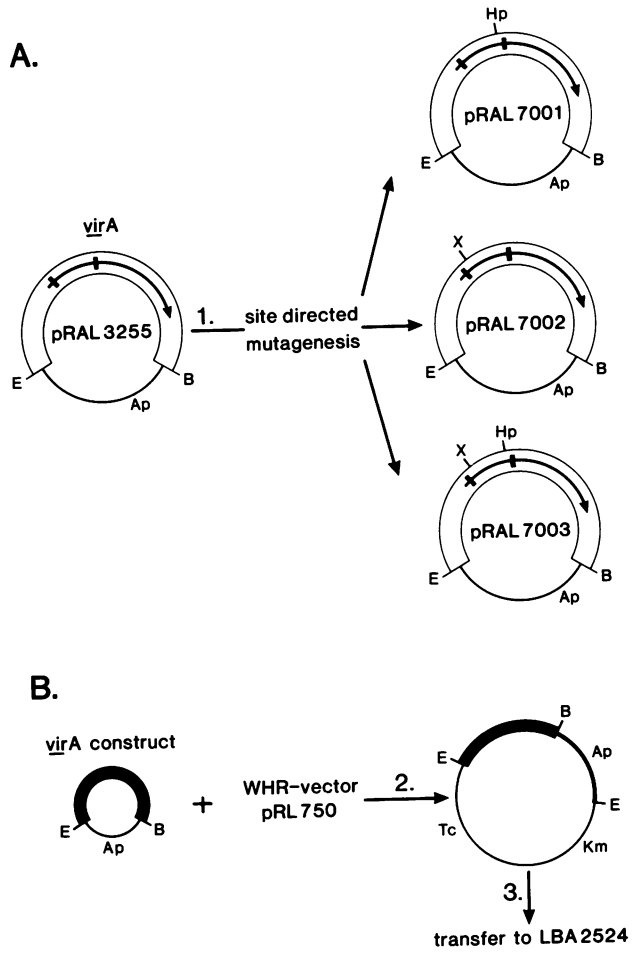

Fig. 3. Construction of *virA*-mutants. (**A**) Plasmid pRAL3255 was modified by site-directed mutagenesis (1) using M13 vectors to create suitable unique restriction sites, *Xba*I and *Hpa*I, without disrupting the reading frame of VirA. (**B**) The *virA* constructs to be tested were cloned into the wide host range (WHR)-vector pRL750 either in the *Eco*RI site as shown or in the *Bam*HI site (2) and were mobilized to strain LBA2524 by conjugation (3). Abbreviations: B, *Bam*HI; E, *Eco*RI; Hp, *Hpa*I; X, *Xba*I.

obtained from three independent mutagenesis experiments, after introduction onto the *incP* class vector pRL750 into the tester strain LBA2524 (see Figure 3B). Characteristic properties of the resulting strains are summarized in Table I. The different constructs tested of each *virA* mutant gave identical results, which suggests that besides the desired modifications no additional mutations were introduced into *virA*. The amino acid substitution (Lys251 → Val251) encoded by *virA*[Hpa] has no significant effect on the signalling properties of VirA. However, the mutants VirA (Ser44 Arg45) and VirA (Ser44 Arg45 Val251), encoded by *virA*[Xba] and *virA*[Xba;Hpa] respectively, were somewhat reduced in their ability to activate *vir* gene expression in response to AS (see Table I). Tumour induction by LBA2524 (*virA*[Hpa]) or LBA2524 (*virA*[Xba]) was similar to that of strain LBA2524 provided with the wild-type *virA* gene in *trans*, whereas the response caused by LBA2524 (*virA*[Xba;Hpa]) was slightly attenuated. Thus, we conclude that these three *virA* point-mutants encode functional proteins and therefore are suitable for the construction of *virA*−*tar* gene fusions and *virA* deletions.

Table I. Effect of amino acid substitutions in VirA on *Agrobacterium vir* induction and tumour induction

Plasmid[a]	Characteristics				Induction	
	Amino acid position				% activity	Tumours on *virB—lacZ N.glauca*
	44	45	251	252		
pRAL3255, wild-type *virA*	Thr	Thr	Lys	Asn	100	+++
pRAL7001, *virA*[Hpa]	Thr	Thr	**Val**	Asn	100	+++
pRAL7002, *virA*[Xba]	**Ser**	**Arg**	Lys	Asn	88	+++
pRAL7003, *virA*[Xba;Hpa]	**Ser**	**Arg**	**Val**	Asn	46	++

[a]Plasmids were cloned into *incP* class vector pRL750 and introduced into strain LBA2524. Amino acid substitutions are in bold type. Wild-type activity was set at 100%.

Functional analysis of VirA−Tar hybrid proteins

We constructed seven chimeric genes coding for VirA−Tar hybrid proteins in which regions from the periplasmic domain and transmembrane domains (TM1, TM2) of VirA were exchanged for those from Tar. It has to be noted here that the topology of the transmembrane protein Tar of *E.coli* is similar to that of VirA. Signalling functions of the VirA−Tar hybrids were monitored by analysis of *vir* induction and tumorigenicity of strain LBA2524 containing the corresponding chimeric genes (numbered 1−7, see Figure 4). All VirA−Tar hybrid proteins contain the C-terminal region of VirA, comprising the region of 200 amino acids which is conserved among a family of sensor proteins and might be involved in signal propagation. The original gene constructs, *virA* (pRAL7030) and *tar* (pRAL7031), were used in these experiments as a positive and negative control, respectively (Figure 4). In agreement with the specificity of the *vir* induction process, we found that the *tar* gene is unable to complement the *virA* mutant strain LBA2524. The Tar protein of *E.coli* recognizes specifically aspartate and maltose-binding protein by its periplasmic domain and effects positive chemotaxis of *E.coli* towards these compounds. Analogous with this, the periplasmic domain of VirA was thought to play an important role in detection of acetosyringone by *Agrobacterium*. In contrast to this model, however, we found that strains with a type 1 *virA*−*tar* fusion, in which the periplasmic domain of VirA is exchanged for that of Tar, still responded to AS. In fact, the effect of AS has become stronger since both the AS-dependent *vir* induction was significantly enhanced and, moreover, tumours were formed more rapidly compared to strain LBA2524 (*virA*). *Agrobacterium* strains with *virA*−*tar* type 2 or type 3 constructs have attenuated signalling properties towards AS, but are still able to provoke tumours on *N.glauca*. It is important to note here that the expression of the type 2, type 3, type 4 and type 7 gene fusions in LBA2524 depends on the activity of the *E.coli tar* promoter in *Agrobacterium*, whereas the other *virA*−*tar* gene fusions are under control of the *virA* promoter. The comparison of the β-galactosidase enzyme activity of a *tar*−*lacZ* (pRAL7046) and a *virA*−*lacZ* (pRAL7047), both translational gene fusions, in *Agrobacterium* showed that the *tar* promoter activity is only 8% of the *virA* promoter activity (data not shown). Therefore, it is mostly likely that the attenuated response as observed with type 2 and type 3 gene fusions is due to the relatively low *tar* promoter activity in

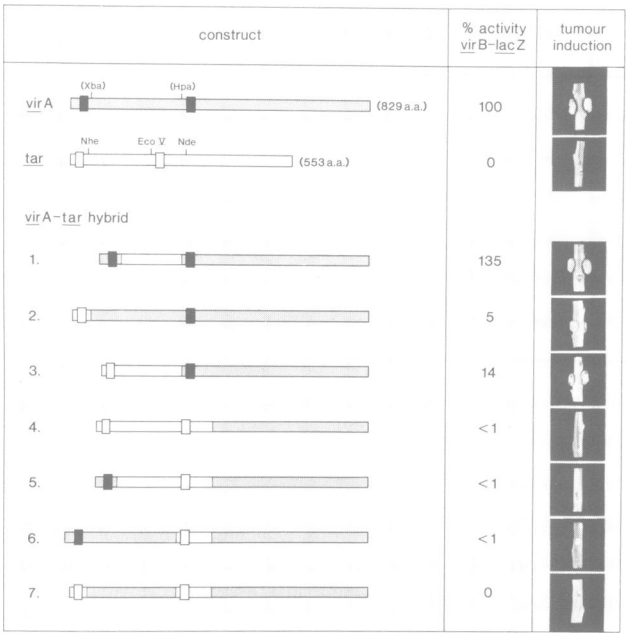

Fig. 4. Activity of *virA−tar* chimeric genes in *Agrobacterium*. The *virA−tar* fusions were tested in strain LBA2524, i.e. AS-induced *vir* gene expression (after 24 h) and tumour induction on *N.glauca*. The VirA−LacZ β-galactosidase enzyme activity of the control strain LBA2524 (pRAL7030) was set at 100%. Open bars represent coding regions of *tar* and hatched bars correspond to *virA* coding regions. The sequences which code for the hydrophobic regions (TM1 and TM2) are boxed. Restriction sites *Xba*I, *Hpa*I, *Nhe*I, *Eco*RV and *Nde*I are shown and were used for cloning the different gene fusions.

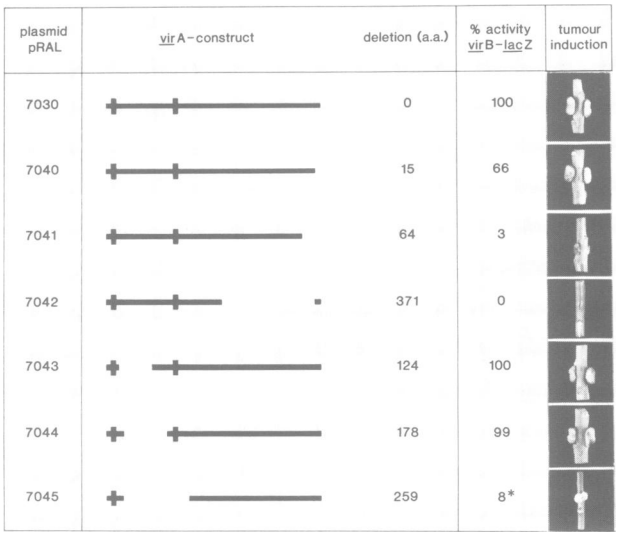

Fig. 5. Activity of *virA* deletion mutants in *Agrobacterium*. The *virA* deletion mutants, pRAL7040 to pRAL7045, including pRAL7030 (control) were tested in *Agrobacterium*, see also legend to Figure 4. VirB−LacZ β-galactosidase activities of the strains were all dependent on the *vir* inducer AS, except for strain LBA2524 (pRAL7045), marked with a star (*), see text. The gaps indicate the internal deletions present within the coding region of *virA*.

Agrobacterium. Comparison of the properties of VirA−Tar type 2 and type 3 hybrids underscores that the presence of a periplasmic domain of Tar positively affected the signalling properties of VirA−Tar hybrids. Furthermore, our results revealed that the N-terminal part of 251 amino acids of VirA is not involved in signal specificity of the regulatory system towards AS. However, substitution of the second transmembrane domain (TM2) of VirA for an analogous region of Tar rendered strain LBA2524 unable to respond to AS. Strains carrying either of the types 4, 5 or 6 *virA−tar* constructs displayed a low constitutive level of *vir* gene expression, which was unaffected by AS, and were able to induce only tiny tumours infrequently (see Figure 4). Reconstruction of the type 4 gene construct with different parts of the N-terminal *virA* coding region created the gene fusions types 5, 6 and 7. Modifications as present in type 5 and type 6 gene fusions had no significant effect on the signalling properties of VirA−Tar type 4 hybrids (see above). Strains provided with a type 7 gene fusion were non-responsive to AS and avirulent on plants. Altogether, these results point to an important role of the TM2 region of VirA in determining the specificity of this signal transducer.

Functional analysis of VirA deletion mutants

In order to characterize further the subdomains of VirA we constructed *virA* mutants that contained specific deletions in their coding region and studied the effect on virulence and the induction of *vir* genes. One deletion mutant (pRAL7043) was constructed via plasmid pRAL7002 (*virA*[Xba]) whereas the other mutants were derivatives of the wild-type *virA* gene (pRAL3255). Results of analysis of *vir* induction and tumour induction behaviour of agrobacteria carrying these constructs are presented in Figure 5. The truncated transducers VirAΔ15 and VirAΔ64 retained 66 and 3% respectively of their ability to stimulate *vir* gene expression in response to AS. In agreement herewith are the virulence properties of the corresponding strains LBA2524(pRAL7040) and LBA2524(pRAL7041), see Figure 5. Strain LBA2524 containing plasmid pRAL7042 displayed no AS-inducible *vir* gene expression and remained avirulent. Thus, small deletions at the C terminus of VirA already affect VirA activity, whereas removal of a large part of the cytoplasmic domain, including the 200 amino acid conserved region, totally abolished its function. Mutant strains carrying the *virA* constructs pRAL7043 or pRAL7044, having a large internal deletion in the coding region of the periplasmic VirA domain, turned out to be normally responsive to AS and were able to induce wild-type tumours. This observation confirms our conclusion drawn earlier from the *virA−tar* experiments, that the entire periplasmic domain of VirA is not important for a sensory function in AS-induced *vir*-gene expression. However, deletion of both the periplasmic region and TM2 region of VirA created the mutant strain LBA2524(pRAL-7045), which is unable to respond to AS but still showed *vir* gene expression up to 8%. This constitutive expression was still pH dependent as no activity at all was found at pH 6.8 instead of the routinely used pH 5.3. Probably this weak constitutive VirA activity depends on a high level of *virG* expression which is inducible at low pH. The tumour response induced by LBA2524 (pRAL7045), as shown in Figure 5, was found only if this strain was grown at low pH conditions prior to the inoculation on *N.glauca*. Thus, these results indicate that the C-terminal VirA part of 508 amino acids comprises a signalling domain which is able to modulate the expression of the *vir* genes. In addition, our data strongly suggest that signal specificity of VirA is linked to its second hydrophobic domain (TM2).

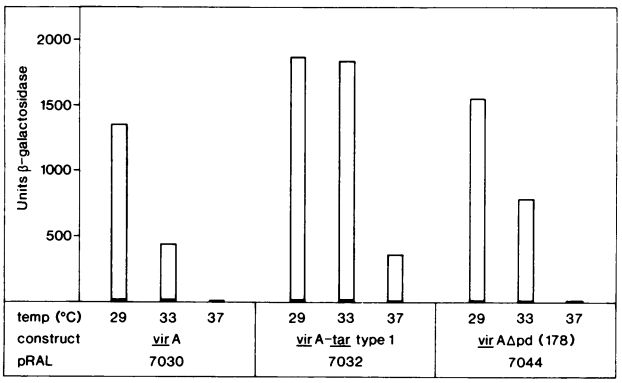

Fig. 6. Properties of *virA* mutants in *Agrobacterium vir* induction tested at different temperatures. VirB−LacZ β-galactosidase activities of the different *Agrobacterium* strains were determined, after growth in induction medium for 24 h, with no inducer (black bars) or with 100 μM AS (open bars) at 29, 33 and 37°C.

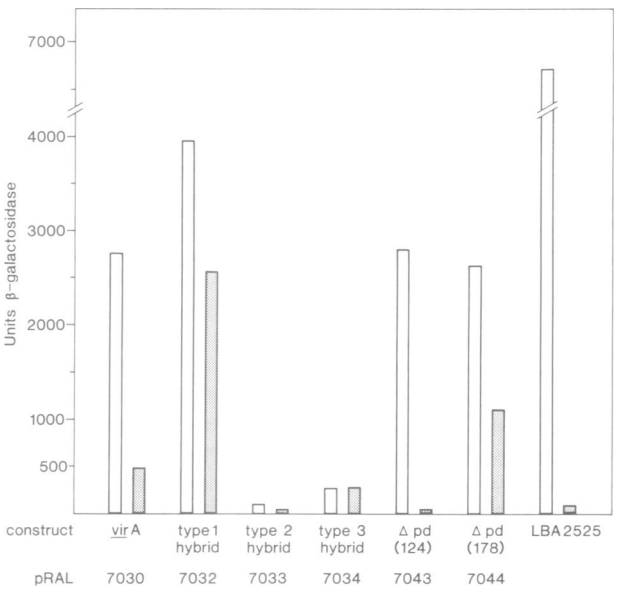

Fig. 7. Properties of *virA* mutants in *Agrobacterium vir* induction at pH 5.3 and pH 6.8. VirB−LacZ β-galactosidase activity of each strain is given for pH 5.3 (open bar) and pH 6.8 (hatched bar) as found after 24 h exposure to 100 μM AS. The black bar at the bottom represents the background enzyme activity that was found without the inducer. Control strains LBA2524 (pRAL7030) and LBA2525 differ only in the in *trans* or in *cis* position of *virA* towards the Ti plasmid respectively.

The role of VirA in temperature and pH sensitivity of vir induction

To explore further the properties of the *virA−tar* hybrid genes and *virA* deletion mutants they were tested for *vir* induction at different conditions, i.e. pH and temperature. It has been shown previously that a temperature sensitive step is involved in the activation of the *Agrobacterium vir* genes (Alte-Moerbe *et al.*, 1988; Melchers *et al.*, 1989). Three types of strains were tested after incubation in IM (− or + AS) at different growth temperatures, 29, 33 and 37°C for 24 h, for the activation of *vir* gene expression, see Figure 6. A characteristic temperature-dependent response was found for both the mutant LBA2524(*virA*Δpd(178)) and the control strain LBA2524(*virA*). These strains display a maximum response at 29°C, which decreased to ~25% at 33°C and became zero at 37°C. Interestingly, strain LBA2524(*virA−tar* type I) displayed an entirely different response to AS at high temperatures as *vir* gene expression was still maximum at 33°C and even significantly induced by AS at 37°C.

Another characteristic feature of *Agrobacterium vir* induction is the requirement for a low pH (pH 5.3 for octopine Ti strains). Our interest was to determine whether an alteration in the VirA amino acid sequence could change this specific feature of *Agrobacterium*. Strains containing the constructs shown in Figure 7 were tested for *vir* induction simultaneously at pH 5.3 and pH 6.8. It was not surprising to find the best induction results at pH 5.3 for all strains. However, strains containing either of the *virA* constructs, *virA−tar* type 1, *virA−tar* type 3 or *virA*Δpd(178) were significantly less sensitive to pH 6.8 in comparison with the control strains LBA2524 (*virA*, in *trans*) and LBA2525 (*virA*, in *cis*). Comparison of the structures and properties of the VirA mutant proteins encoded by pRAL7043 and pRAL7044 suggests that the pH sensitivity of the *vir* induction process is in part determined by a 73 amino acid region (position 169−241) of the periplasmic domain of VirA. Furthermore, it is most likely that the amino acids of the Tar periplasmic domain as present in VirA−Tar type 1 hybrids are responsible for the increased ability of *Agrobacterium* to respond to AS at higher temperatures.

Discussion

The VirA protein is a membrane protein required for the transcriptional regulation of *Agrobacterium* virulence genes. In this study, we investigated the roles of VirA in both signal transduction and mediating a cellular response to specific plant signal molecules like AS. The primary structure of VirA has been deduced from sequence data and suggests that VirA comprises a number of discrete structural domains analogous to chemosensory transducers of *E.coli* like Tar and Tsr (Leroux *et al.*, 1987; Melchers *et al.*, 1987). Functional analysis of these chemoreceptors of *E.coli* revealed that specific functions as receptor domain and signalling domain are determined by the periplasmic domain and cytoplasmic domain of the molecule respectively.

We first analysed the membrane topology of VirA using fusions to alkaline phosphatase (Manoil and Beckwith, 1986) which were tested in *E.coli*. Only hybrid proteins with alkaline phosphatase fused to the periplasmic domain of VirA showed high PhoA activity. An exception was found for one active VirA−PhoA fusion, which has its junction within the second transmembrane sequence of VirA (position 270). This finding suggests that the remaining 11 amino acids of the second hydrophobic region are unable to anchor this fusion protein in the membrane and to hold the AP moiety in the cytoplasm (Adams and Rose, 1985). Summarizing, our results confirm that VirA possesses a simple transmembrane structure (see Figure 2) as predicted from the VirA amino acid sequence as deduced from its DNA sequence data. At present it is unknown whether the N-terminal hydrophobic region is removed from VirA by cleavage of the signal sequence upon transport across the cytoplasmic membrane or not. Studies on the transducers Tar, Tsr, CpxA and EnvZ of *E.coli* revealed that their signal sequences are not removed

from the mature polypeptide and are thought to anchor the N-terminus in the membrane (Mowbray *et al.*, 1985; Forst *et al.*, 1987; Gebert *et al.*, 1988; Weber and Silverman, 1988).

Secondly, we used a genetic approach to define the functions of the distinct VirA subdomains (periplasmic domain, cytoplasmic domain, hydrophobic domains) in transmembrane signalling. We introduced several types of mutations into the *virA* coding region and studied their effect on AS-stimulated *vir* gene expression. The exchange of the periplasmic domains of VirA and Tar, or even a complete deletion of this VirA subdomain had no effect on transmembrane signalling. Interestingly, VirA−Tar type 1 hybrids containing the periplasmic region of Tar, are strongly enhanced in their ability to stimulate *vir* gene expression in response to AS, and to transform *N.glauca* leaf discs (unpublished results). The signalling properties of the type 2 and type 3 VirA−Tar hybrids, which contain alterations at the N terminus of VirA, are somewhat attenuated but these proteins can still mediate *Agrobacterium vir* induction in response to AS. These results clearly show that a putative receptor domain of VirA for AS must be located somewhere beyond its periplasmic domain. We propose that the second transmembrane region of VirA is involved in stimulus detection other than merely serving as a membrane anchor and that the C-terminal part of VirA comprises a cytoplasmic signalling domain. This hypothesis is supported by the following observations. *Agrobacterium* strains coding for VirA proteins that lack their TM2 region or contain the TM2 region of Tar instead, are unable to respond to AS. These modifications do not completely abolish the signal output of VirA, since low levels of *vir* gene expression are observed and these strains can occasionally form small tumours on *N.glauca*. The VirA protein might interact with AS in a manner similar to that by which ligands interact with the β-adrenergic receptor, i.e. by binding to the hydrophobic transmembrane region of this eukaryotic receptor (Strader *et al.*, 1987). The signalling function of VirA is negatively affected by small deletions at the C terminus and totally absent if a large deletion is present in the cytoplasmic domain. The carboxyl termini of a family of prokaryotic sensors are similar in sequence over a length of ~200 residues (Ronson *et al.*, 1987). This region could represent a signalling domain that mediates signal transmission possibly by protein−protein (i.e. VirA−VirG) communication. In conclusion, the location of a putative signalling function within the cytoplasmic domain of VirA would be in agreement with its cellular location in *Agrobacterium* and analogous to the chemosensory transducers of *E.coli*.

Shaw *et al.* (1988) reported that *virA* and *virG* are required for chemotaxis of *A.tumefaciens* towards AS. They proposed a multifunctional role for VirA, i.e. at low concentrations of AS (10^{-8} M) VirA mediates chemotaxis and at higher concentrations (10^{-6} M) it effects *vir* induction. It would be interesting to determine whether the periplasmic domain of VirA plays a role in the chemotaxis of *Agrobacterium* towards AS or is not essential at all, as shown for *vir* induction.

So far, we are able to assign two characteristic properties of *vir* induction, namely 'pH sensitivity' and 'temperature sensitivity', at least in part, to the VirA protein of *Agrobacterium*. We show here that the exchange of the periplasmic domains of VirA and Tar creates a hybrid protein that is less 'thermosensitive'. Since the removal of the periplasmic domain of VirA does not alter its thermosensitive property, we propose that the periplasmic Tar region of the VirA−Tar type 1 hybrid changes the conformation of the signal transducer and accounts for both its altered temperature-sensitive and pH-sensitive properties regarding *vir* induction. Moreover, the pH sensitivity is linked to a region (amino acid positions 169−241) close to the TM2 region of VirA, suggesting that the external pH might control the conformation of VirA in this region, and renders the putative receptor in TM2 active (at pH 5.3) or inactive (at pH 6.8).

The VirA model described in this article provides a new, but still incomplete, view of the structure−function organization in VirA. However, it is possible now to apply more refined genetic approaches, such as site-directed mutagenesis of TM2, to obtain more information on the transmembrane signalling mechanism of VirA.

Materials and methods

Materials

Restriction endonucleases were purchased from Pharmacia and used according to the supplier's recommendations; the Sequenase DNA sequencing kit was from US Biochemical Corp. and the following oligodeoxynucleotides were purchased from MOGEN Int. Leiden: (*Xba*)-26mer, 5′-GGATTGC-CTGTCTAGACGCATTGTCC-3′; (*Hpa*)-26mer, 5′GCTCCTCTACGTT-AACCAAGCTATAG-3′; (*Pho*A3)-17mer, 5′-CGCTAAGAGAATC-ACGC-3′; *Nde*-16mer, 5′-TATGATTCTGCAGCGC-3′ and C-10mer, 5′-TGCAGAATCA-3′ (complementary to the *Nde*I-16mer). [γ-^{32}P]ATP and [α-^{32}P]dATP were purchased from New England Nuclear, phenylmethylsulfonylfluoride (PMSF) from Sigma and acetosyringone (AS) was from EGA-chemie.

Media, growth conditions and enzyme assays

Growth media, LC, MM and induction medium (IM) have been described elsewhere (Hooykaas *et al.*, 1979; Melchers *et al.*, 1989). Unless indicated otherwise, *E.coli* strains were grown at 37°C, and *Agrobacterium* strains at 29°C.

Antibiotic concentrations (μg/ml) for *E.coli* strains were: carbenicillin (Cb), 100; kanamycin (Km), 25; tetracyclin (Tc), 10; and for *Agrobacterium* strains: Cb, 100; Km, 100; rifampicin (Rif), 20; spectinomycin (Sp), 250. 5-Bromo-4-chloro-3-indolylphosphate (XP) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were added to media at final concentrations of 40 and 20 μg/ml respectively.

Alkaline phosphatase assays of late log-phase *E.coli* cells, grown in LC supplied with Cb, were done as follows: 1 ml of cells was pelleted in a microcentrifuge, washed twice with 1 ml of 10 mM Tris−HCl (pH 8.0), 0.5 mM PMSF and resuspended in 1 ml of 1 M Tris−HCl (pH 8.0), 100 μl of 0.4% *p*-nitrophenyl phosphate (Sigma) was added and the reaction was stopped after 30 min with 100 μl of 1 M K_2HPO_4. Cells were pelleted and the OD_{420} was measured in a cell-free supernatants, where the OD_{550} was approximately zero. Units of alkaline phosphatase were calculated as OD_{420} per 10^{10} bacteria.

Agrobacterium vir induction was determined using a *virB2−lacZ* translational fusion that is present in the octopine Ti plasmid of strain LBA2524 (Δ*virA*) and LBA2525. Strains were grown in IM supplied with 100 μM AS and β-galactosidase assays were performed as described previously (Melchers *et al.*, 1989).

Bacterial strains and plasmids

Bacterial strains used were: *E.coli*, CC118 (Δ*phoA*) (Manoil and Beckwith, 1985), KMBL1164(Δ *lac-pro*) (Giphart-Gassler and Van de Putte, 1979), HB2151, HB2154(*mut*L::Tn*10*) (Carter *et al.*, 1985), HB101(pRK2013) (Figurski and Helinski 1979) and *Agrobacterium*, LBA1010 (octopine pTiB6) LBA2524 (Δ*virA*, *virB−lacZ*). Strain LBA2525 is a derivative of LBA1010 and contains a translational *virB2−lacZ* gene fusion integrated in the Ti plasmid. Strain LBA2524 was constructed by deletion of the entire *virA* gene from the Ti plasmid of LBA1010, performed as described previously (Melchers *et al.*, 1987) and recombination of the *virB2−lacZ* gene fusion into the Ti plasmid as described for LBA2525 (Melchers *et al.*, 1989). Plasmid pRAL3255 in pUC19 carrying Ti fragment *Kpn*I−10 (*virA*$^+$) as

shown in Figure 3A. Plasmid pCH2 (Hoffman and Wright, 1985) was kindly provided by Dr Wright. Plasmid pRBB6 is pBR322 which contains the *E.coli tar* gene on a 3.2 kb *Eco*RI−*Ava*I fragment, with a new *Nhe*I site within the *tar* coding region (Val42 → Ala42), whereby the encoded Tar protein is functional (R.B.Bourret, unpublished). Insertion of the Km^r marker (0.9 kb *Pst*I fragment) of pUC-4K (Vieira and Messing, 1982) into pMP92 (Spaink *et al.*, 1987) resulted in the broad-host-range vector pRL750. Plasmid pRL751 is a derivative of pIC19R containing the *phoA* coding region of pCH2 (2.4 kb *Pst*−*Xho*I fragment) preceded by the unique restriction sites *Bgl*II, *Xba*I, *Sma*I, *Xma*I, *Bam*HI, *Sal*I and *Pst*I, all suitable for cloning *virA*−*phoA* gene fusions. Cloning of various *virA* DNA fragments into pRL751 provided the *virA*−*phoA* gene fusions shown in Figure 1. All junction sites were checked by supercoiled DNA sequencing (Chen and Seeburg, 1985) using the *PhoA3* primer, to ensure a correct *virA*−*phoA* reading frame. The plasmids pRAL7046 and pRAL7047 contain the translational gene fusions *tar*−*lacZ* and *virA*−*lacZ* respectively in vector pRL750. The hybrid proteins encoded by these constructs contain at their C terminus the LacZ moiety fused to the cytoplasmic domain of either Tar or VirA.

Site-directed mutagenesis and plasmid constructs

In vitro mutagenesis of *virA* was done essentially by the method of Carter *et al.* (1985). Briefly, the *Eco*RI−*Bam*HI fragment of pRAL3255 containing *virA* was ligated into vector M13mp8 and single-stranded phage DNA was prepared, annealed with *Eco*RI and *Hin*dIII digested vector M13mp18 to form the gap duplex [mp8*virA*−mp18]. The phosphorylated oligodeoxynucleotides *Xba*-mer and *Hpa*-mer were mixed with the gap duplex DNA for annealing, followed by a Klenow and ligation reaction. Recombinant phage M13mp18-*virA* was selected by transformation to *E.coli* strain HB2154 (*mutL*) using HB2151 as lawn. A large number of independent *virA* mutants, containing either of the restriction sites *Hpa*I, *Xba*I, or both, were cloned as *Eco*RI−*Bam*HI fragments into pUC8 to yield pRAL7001, pRAL7002 and pRAL7003 respectively (see Figure 3A). These *virA* constructs, pRAL3255 and pRBB6, were used to create seven different *virA*−*tar* gene fusions as shown in Figure 4. Plasmids pRAL7032 to pRAL7038 correspond to the *virA*−*tar* hybrids types 1−7 respectively. Restriction sites used for cloning were: *Xba*I, *Hpa*I and *Hae*II for *virA* and *Nhe*I, *Eco*RV and *Nde*I for *tar*. The exchange of the various *virA* and *tar* DNA fragments resulted in the chimeric constructs shown in Figure 4. Plasmid pRAL7035 (type 4) was made by ligation of the 1.8 kb *Hae*II fragment of pRAL3255, which contains the 3′ end of *virA* into the *Nde*I site of pRBB6 as drawn in Figure 4 by using a synthetic *Nde*I−*Pst*I−*Hae*II DNA linker (i.e. *Nde*I-16mer annealed with the phosphorylated oligo C-10mer). Plasmids pRAL7036 and pRAL7037 are derivatives of pRAL7035, whereas pRAL7038 was made via pRAL7037. Deletion constructs shown in Figure 5 were made by deleting the following DNA restriction fragment from pRAL3255 or pRAL7002: pRAL7040, Δ*Eco*RV; pRAL7041, Δ*Sal*I; pRAL7042, Δ*Sph*I; pRAL7043, Δ*Xba*I−*Nhe*I; pRAL7044, Δ*Pst*I and pRAL7045, Δ*Pst*−*Hae*II. The 1.8 kb *Pst*I fragment that contains the 3′ end of *virA* was taken from pRAL7035 and used to create pRAL7045. DNA sequence analysis of the *Pst*I cloning site of pRAL7045, established that the *virA* coding regions were fused in the correct reading frame. Each of the *virA*−*tar* hybrids and *virA* deletion constructs was made twice in parallel experiments and was analysed extensively, with at least three different restriction endonucleases to check its contents and to ensure that the orientation of the DNA fragments was correct. These constructs were made initially in a pUC8, or pBR322, vector and finally were cloned into the broad-host-range vector pRL750 (see Figure 3B) to yield pRAL7030 to pRAL7045. Plasmids containing the vector pRL750 were transferred from *E.coli* strain KMBL1164 to *Agrobacterium* by conjugation using the helper strain HB101(pRK2013) for mobilization.

Virulence assay

Tumour induction was tested on stems of *N.glauca* by puncturing the stem with a toothpick containing an inoculum of *Agrobacterium*. All strains were tested twice on at least four different plants. Tumour formation was scored after 14 days.

Acknowledgements

We thank Dr Ron van Veen for critical reading of the manuscript and Mrs Ria Bergenhenegouwen for her excellent secretarial assistance. This work was supported by the Netherland Foundation of Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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Received on March 29, 1989; revised on April 21, 1989