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Localization of the VirA domain involved in acetosyringone-mediated *vir* gene induction in *Agrobacterium tumefaciens*

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Abstract

The VirA protein of *Agrobacterium tumefaciens* is thought to be a receptor for plant phenolic compounds such as acetosyringone. Although it is not known whether the interaction between VirA and the phenolics is direct or requires other phenolic-binding proteins, it is shown in this study that the first 280 amino acids of the VirA protein are not essential for the acetosyringone mediated *vir* gene induction response. Considering the fact that the cytoplasmic region between the amino acids 283 and 304 is highly conserved between the different VirA proteins, and that deletion of this region abolishes VirA activity, we suggest that the acetosyringone receptor domain is located in this cytoplasmic domain of the VirA protein.

Introduction

The products of the *Agrobacterium tumefaciens* virulence genes mediate a process that resembles bacterial conjugation [2] and by which part of the Ti plasmid (T-DNA) is transferred to the plant cell nucleus. The expression of specific T-DNA genes within the plant cell results in the production of plant hormones, such as auxin and cytokinin, which cause cell proliferation and the formation of a tumour. The expression of the virulence genes is controlled by the products of the *virA* and *virG* genes and dependent on the presence of plant phenolic compounds such as acetosyringone. The VirA and VirG proteins are members of a two-component regulatory family

and possess homology to other members such as EnvZ-OmpR and DctB-DctD. In two-component regulatory systems, one component senses environmental signals and activates the second component to carry out a function (for a recent review see [24]). In *A. tumefaciens* the VirA protein is thought to act as the receptor for plant phenolic compounds like acetosyringone. Whether this interaction between the phenolics and the VirA protein is direct or requires other phenolic-binding proteins is not known yet [14]. *In vitro* experiments showed that the cytoplasmic portion of the VirA protein has autokinase activity and is able to phosphorylate the VirG protein [8, 10, 11]. The VirG protein binds to specific sequences in the *vir* promoters ('*vir* box'), and it

is thought that upon phosphorylation the VirG protein becomes an efficient activator of *vir* gene transcription [12, 25, 27]. Sequence analysis of the *virA* gene predicted two hydrophobic regions within the VirA protein which might function as transmembrane regions [15, 18]. Indeed, topology studies with VirA-PhoA fusion proteins revealed that the VirA protein is located in the innermembrane of *A. tumefaciens* [19]. The topology of the VirA protein resembles that of other well-known receptor proteins such as the chemoreceptor Tar, a member of the class of methyl-accepting chemotaxis proteins that mediates chemotaxis towards attractants such as aspartate, maltose and phenol and away from repellents such as Ni^{2+} and glycerol [6, 9, 19]. For the chemoreceptor Tar it is known that the periplasmic domain plays an important role in ligand sensing, while the two transmembrane regions are thought to play an important role in the maintenance of protein conformation [23]. Studies with VirA-Tar chimeric proteins revealed that the periplasmic domain of the VirA protein is not essential for induction by phenolic compounds [19]. The periplasmic domain, however, is essential for the enhancement of *vir* gene induction by monosaccharides such as *D*-glucose [3, 29]. It is thought that the ChvE protein, which is homologous to the *Escherichia coli* galactose-binding protein, is able to bind monosaccharides and to enhance *vir* gene induction by binding to the periplasmic domain of VirA, thereby changing its conformation. This is confirmed by the observation that exchanging the periplasmic domain of VirA for that of Tar results in a protein which is non-responsive to sugars but locked in a highly responsive state which mimics the ChvE-occupied receptor conformation [31]. Melchers *et al.* [19] suggested that the receptor domain for the phenolic compounds might be located within, or in the vicinity of the second transmembrane domain. Hybrid proteins in which a region, containing the second transmembrane domain and the neighbouring cytoplasmic portion, was altered were not able to respond to the phenolic inducers. Recently, it was shown that a VirA deletion mutant which lacked the entire N-terminal part of

the protein, including the two transmembrane regions, still had the ability to induce a *virB-lacZ* reporter construct in the presence of 100 μM acetosyringone [4]. This indicated that neither of the two transmembrane domains is essential for the induction by phenolic compounds. Pazour *et al.* [26], however, reported that changing a leucine residue, at position 24 in the first transmembrane region, into a phenylalanine residue resulted in elevated *vir* gene expression in the absence of phenolic compounds. This indicated that the first transmembrane region plays an important role in determining the protein conformation which is essential for optimal VirA activity. In order to localize the phenolic receptor domain and to elucidate the role of the different regions in the activation of the VirA protein by phenolic compounds, several mutant proteins were constructed. In this report we confirm that the periplasmic and the two transmembrane regions are dispensable for the induction by phenolic compounds.

Materials and methods

Materials

Restriction endonucleases were purchased from either Promega Biotec. or New England Biolabs and used according to the suppliers' recommendations. The oligonucleotides used are listed in Table 1. *O*-Nitro- β -*D*-galactopyranoside and uridine were purchased from Sigma Chemical Co. and acetosyringone (AS) from EGA-chemie.

Bacterial strains and plasmids

Bacterial strains used were: *Agrobacterium tumefaciens* LBA2524 (pTiB6: Δ *virA*,*virB-lacZ*) [19] and *E. coli* JM101 [34] and PP1674 (*dut*,*ung*) [33].

In order to generate VirA-Tar chimeric proteins, specific restriction sites were introduced into the coding region of the *virA* gene (Fig. 1). Plasmid pST7111 was constructed by cloning a 4.5 kb *Eco*RI-*Bam*HI fragment of pRAL3254 [19], containing the complete *virA* gene of

Table 1. Oligonucleotides used in this study. Nucleotide substitutions are in bold type and restriction sites are underlined.

Mutagenesis

22-mer (<i>Bss</i> HII)	5'-GGCTTC <u>GCGCGCGTCTT</u> AAAAT-3'
30-mer (<i>Bss</i> HII)	5'-CCCAAGAAAGAT <u>GCGCGCGCTCCGCTC</u> TC-3'
33-mer (<i>Sal</i> I)	5'-TAACCAATCGGTTC <u>GTCGAC</u> GTAGCCTATAGAC-3'
21-mer (<i>Bst</i> BI)	5'-CTCACCTTCGAAACATACTCC-3'

PCR

26-mer (<i>Bss</i> HII)	5'-ATTAAGCGCGCCCGCGTAGTCACGCT-3'
23-mer	5'-CATCATCCGTACCGCTGAACGAC-3'
30-mer (<i>Bss</i> HII)	5'-CAAAGCGCGCGTATCTTTGCCAGTGCGAA-3'
32-mer (<i>Sal</i> I)	5'-GTTCTGTCGACGTAGCCTAATGCCGTACACGC-3'

pTi15955, into *Eco* RI-*Bam* HI-digested pTZ18R (USB corporation, Cleveland). The *virA* gene of pRAL7003 [19] was mutagenized by the method

of Kunkel *et al.* [13]. A 22-mer oligonucleotide was used to introduce a *Bss* HII restriction site in the *virA* gene. Due to the introduction of the

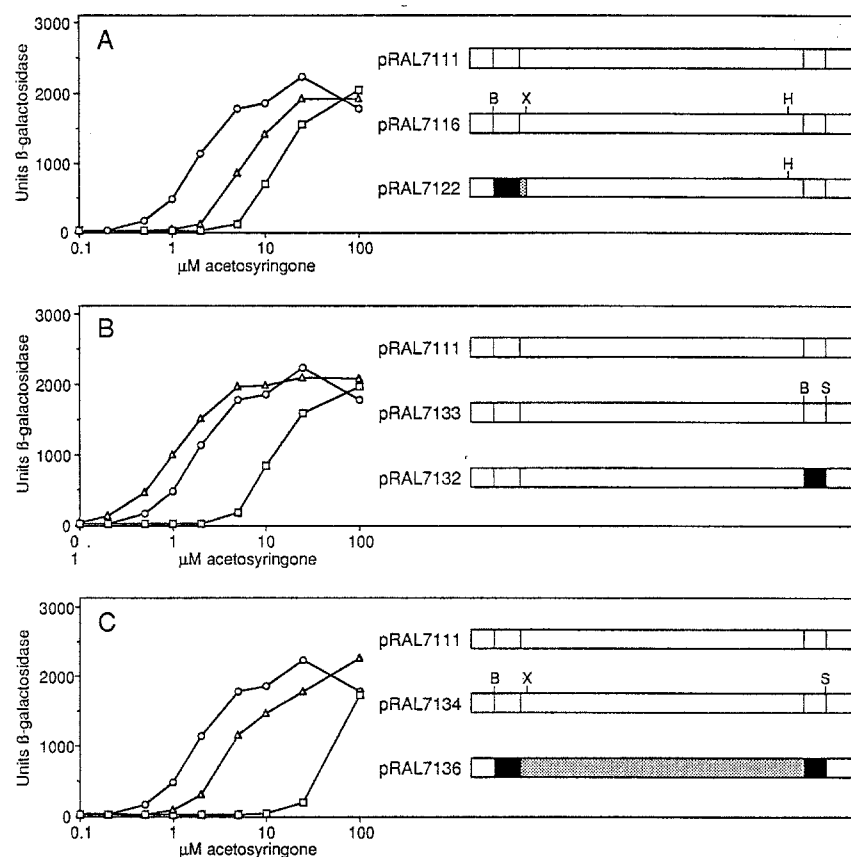


Fig. 1. Induction characteristics of *virA-tar* chimeric genes in *Agrobacterium*. The *vir* gene induction response of LBA2524 harbouring a wild-type *virA* gene (○), a *virA* gene containing several restriction sites (△) or the *virA-tar* chimeric genes (□) was tested in induction medium with 10 mM glucose and various concentrations of acetosyringone. Flasks were shaken for 18 h at 28 °C and units β-galactosidase were determined as described [20]. Open bars represent coding regions of *virA*, black and stippled bars represent coding regions of *tar*. The restriction sites *Bss* HII (B); *Xba* I (X); *Hpa* I (H) and *Sal* I (S) shown were used for the construction of the different *virA-tar* gene fusions.

Bss HII restriction site the amino acid glycine at position 15 in the *VirA* protein was changed into arginine. Subsequently a 4.5 kb *Eco* RI-*Bam* HI fragment, containing the mutagenized *virA* gene, was cloned into *Eco* RI-*Bam* HI-digested pTZ18R yielding pST7116. Using a 26-mer (*Bss* HII) oligonucleotide and a 23-mer oligonucleotide, a 180 bp fragment was amplified by PCR from pRBB6 [19]. This PCR fragment, coding for the first transmembrane region of the protein Tar, was digested with *Bss* HII and *Nhe* I and cloned into the *Bss* HII-*Xba* I digested pST7116, yielding pST7122. Plasmid pST7133 was constructed by mutagenizing plasmid pST7111. Using a 30-mer (*Bss* HII) oligonucleotide and a 33-mer (*Sal* I) oligonucleotide, a *Bss* HII and *Sal* I restriction site were introduced into the *virA* gene of pST7111. Due to the introduction of the *Sal* I restriction site the amino acids Lys-282 and Lys-283 were changed into Arg-282 and Arg-283. For the construction of plasmid pST7132 a 80 bp PCR fragment, coding for the second transmembrane region of the protein Tar, was amplified from pRBB6 using the 30-mer (*Bss* HII) oligonucleotide and the 32-mer (*Sal* I) oligonucleotide. This fragment was digested with *Bss* HII and *Sal* I and cloned into the *Bss* HII-*Sal* I-digested pST7133 to yield pST7132. Plasmid pST7134 was constructed by cloning a 1.2 kb *Nhe* I-*Sna* BI fragment of pST7137 into *Nhe* I-*Sna* BI-digested pST7116. For the construction of pST7136 a 600 bp PCR fragment, coding for the N-terminal part of the protein Tar, was amplified from pRBB6 using the 26-mer (*Bss* HII) oligonucleotide and the 32-mer (*Sal* I) oligonucleotide. This PCR fragment was digested with *Bss* HII-*Sal* I and cloned into *Bss* HII-*Sal* I-digested pST7134 to yield pST7136. Plasmid pST7137 was constructed by introducing a *Sal* I and *Bst* BI restriction site into the *virA* gene of pST7111 using a 33-mer (*Sal* I) and a 21-mer (*Bst* BI) oligonucleotide. Deletion constructs were made by digesting pST7137 with the following restriction enzymes: pST7138, Δ *Sal* I-*Bst* BI; pST7139, Δ *Bst* BI-*Eco* 47III; pST7140, Δ *Sal* I-*Eco* 47III. The restriction sites were filled in by the Klenow fragment of DNA polymerase, followed by ligation.

To verify the DNA sequence, all DNA fragments containing mutations and PCR generated DNA fragments were sequenced by the chain termination method [28].

Plasmids pST7111 to pST7140 were linearized with *Eco* RI and cloned into the unique *Eco* RI restriction site of the IncP class vector pRL750 [19], resulting in pRAL7111 to pRAL7140, respectively. Cointegrates were transferred to *A. tumefaciens* by electroporation [17].

Media and antibiotics

E. coli strains were grown at 37 °C in LC medium [7] and *A. tumefaciens* strains at 29 °C in minimal medium (MM) [7]. Induction medium for *A. tumefaciens vir* induction (IM) contained MM salts, 62.5 mM potassium phosphate pH 5.3 and 0.5% glycerol as a carbon source. Antibiotic concentrations (μ g/ml) for *E. coli* strains were: carbenicillin, 100; kanamycin, 25; tetracyclin, 10; and for *A. tumefaciens*: carbenicillin, 75; kanamycin, 100; tetracyclin, 2; rifampicin, 20; spectinomycin, 250.

Induction of *Agrobacterium tumefaciens*

Bacteria were grown overnight in MM to an optical density (OD₆₀₀) of 0.7–0.8. Cells were pelleted by centrifugation and diluted 1:20 in IM with various concentrations of acetosyringone (stock solution in DMSO). 10 mM glucose was supplemented as indicated. Flasks were shaken for 18 h at 28 °C and β -galactosidase activity was measured as described [20].

Assays were repeated at least three times in which similar data were obtained.

Results

Effects of introduced restriction sites on *VirA* signalling

In order to find out whether the two transmembrane regions play a role in acetosyringone recognition and/or signal transduction, the properties of several *virA-tar* hybrid genes were studied.

Therefore, genes were constructed coding for chimeric proteins of which (1) the first transmembrane region, (2) the second transmembrane region, or (3) the entire N-terminus of the VirA protein were precisely exchanged for the corresponding region of the *E. coli* chemoreceptor Tar.

To be able to construct the *virA-tar* hybrid genes, several unique restriction sites were first introduced into the coding region of the *virA* gene [19, 31]. It had been determined previously that the introduction of the *Xba* I restriction site resulting in the VirA(Ser-44, Arg-45) mutant affected the functioning of VirA, i.e. the mutant protein no longer responded to sugars and the ChvE protein and was locked in a low responsive state [31]. Strains containing the VirA(Arg-15) protein, encoded by the *virA(Bss* HII) gene or the VirA(Val-251) protein, encoded by the *virA(Hpa* I) gene, however, showed the same induction pattern as a strain containing a wild-type VirA protein [31]. Plasmid pRAL7133 contains a *virA* gene in which a *Sal* I and *Bss* HII restriction site had been introduced and codes for the VirA(Arg-282, Arg-283) protein. Introduction of the *Bss* HII site in the *virA* gene did not lead to alterations in the VirA protein sequence. To determine the effects of the amino acid alterations caused by the introduction of the *Sal* I restriction site, plasmid pRAL7133 was introduced into *A. tumefaciens* strain LBA2524 and *vir* gene induction was measured. Figure 1b shows that LBA2524(pRAL-7133) had a similar *vir* gene induction response as LBA2524(pRAL7111), which harbours a wild-type VirA protein, indicating that the amino acid alterations caused by the introduction of the *Sal* I restriction site have no negative effect on the *vir*-gene induction response.

Role of the membrane-spanning regions

To determine the role of the different VirA domains in signal perception and transduction pRAL7122, pRAL7132 and pRAL7136 were, together with several controls, introduced into *A. tumefaciens* strain LBA2524. In this *A. tumefaciens* strain the entire *virA* gene is deleted and a *virB-lacZ* reporter gene is present in order to

monitor *vir* gene expression [19]. The *vir* gene induction response of the resulting strains was determined in the presence of 10 mM *D*-glucose and various concentrations of acetosyringone. Figure 1 shows that in the presence of 100 μ M acetosyringone all strains showed high *vir* gene induction. This indicates that the first 280 amino acids of the VirA protein are not essential for the recognition of acetosyringone. At lower concentrations of acetosyringone, however, differences in the induction response patterns of the different strains were observed. In the presence of 5 μ M acetosyringone LBA2524(pRAL7122) and LBA2524(pRAL7132), which harbour chimeric proteins of which respectively the first or the second transmembrane region of VirA was exchanged for that of Tar, showed only 6% *vir* gene induction activity as compared to a strain harbouring a wild-type VirA protein. Exchanging the entire N-terminus of the VirA protein by that of Tar resulted in a chimeric protein which was non-responsive to the presence of 5 μ M acetosyringone. To obtain a weak *vir* gene induction response at least 25 μ M acetosyringone needed to be present in the induction medium in the case of LBA2524(pRAL7136).

Sugars like *D*-glucose are able to enhance the *vir* gene induction response in wild-type strains [3, 29]. Determining the responsiveness of the three strains, containing the VirA-Tar chimeric proteins, to ChvE inducing sugars revealed that LBA2524(pRAL7122), LBA2524(pRAL7132) and LBA2524(pRAL7136) are no longer responsive to sugars and the ChvE protein (data not shown). Testing the responsiveness of the three strains towards the natural inducers of the Tar protein, revealed that neither aspartate (1 mM) nor phenol (200 μ M) was able to induce the virulence genes in LBA2524 harbouring pRAL7122, pRAL7132 or pRAL7136 (results not shown).

Tumour response of the strains harbouring the chimeric proteins

Figure 1 shows that exchanging different domains of the VirA protein by those of the chemorecep-

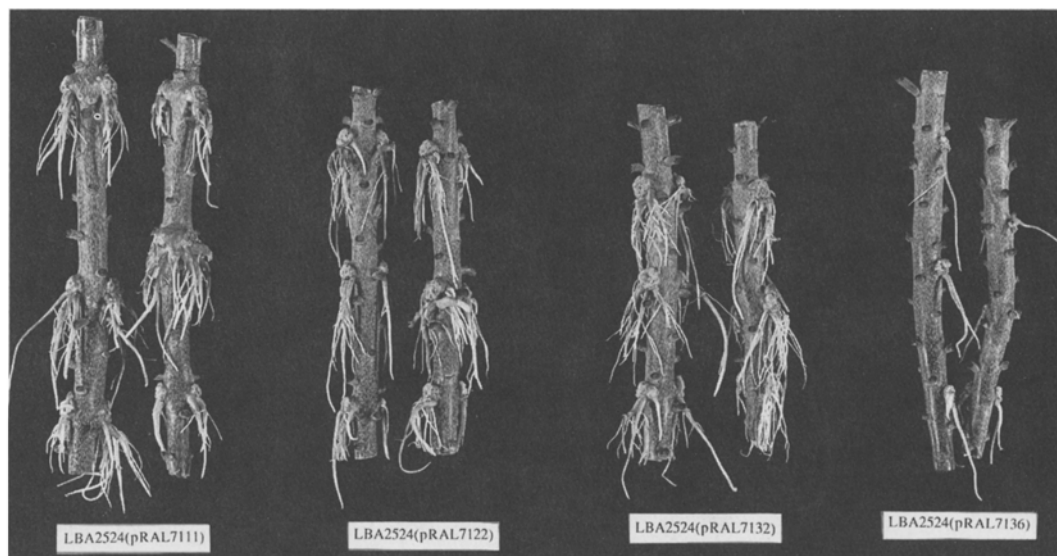


Fig. 2. Tumour response of the *virA-tar* chimeric genes in *Agrobacterium tumefaciens* strain LBA2524 upon infection on stems of *Kalanchoe tubiflora*. Tumour formation was evaluated after 3 weeks.

tor Tar resulted in chimeric proteins which are still able to respond to acetosyringone, although the amount of acetosyringone needed to provoke a *vir* gene induction response was higher than observed with a wild-type strain. To find out whether strains harbouring these proteins were still able to provoke tumours LBA2524(pRAL7122), LBA2524(pRAL7132) and LBA2524(pRAL7136) were inoculated on *Kalanchoe tubiflora*. Figure 2 shows that all strains were able to provoke tumours as expected, although the tumours induced by LBA2524(pRAL7136) were smaller in size than those formed by the wild-type strain LBA2524(pRAL7111).

Deletion analysis of the *VirA* protein

Melchers *et al.* [19] speculated that the receptor domain for the phenolic compounds might be located within or in the vicinity of the second transmembrane region. Figure 1 shows that the N-terminal part of the *VirA* protein, including the second transmembrane domain, is not essential for the induction by phenolics. In order to determine the role of the cytoplasmic linker domain in

the acetosyringone mediated *vir* gene induction response and in an attempt to localize the receptor domain for the phenolic compounds, several deletion constructs were created (Fig. 3). Testing the responsiveness of these mutant strains to acetosyringone revealed that none of the mutants harbouring the plasmids pRAL7138, pRAL7139 or pRAL7140 was able to induce the *virB-lacZ* reporter construct in the presence of acetosyringone (Table 2). This could indicate either that the cytoplasmic region close to the second transmembrane region is essential for the induction by acetosyringone or that the deletions lead to an altered *VirA* protein conformation, resulting in

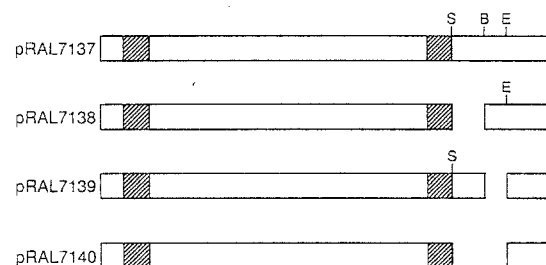


Fig. 3. Schematic representation of the *VirA* deletion mutants. The restriction sites *Sal* I (S), *Bst* BI (B) and *Eco* 47III (E) used to create the deletions are indicated.

Table 2. Effect of deletions on the acetosyringone-mediated *vir* gene induction response.

Strain/plasmid	– AS	100 μ M AS
LBA2524(pRAL7137)	31	1928
LBA2524(pRAL7138)	24	33
LBA2524(pRAL7139)	33	35
LBA2524(pRAL7140)	25	25

mutant VirA proteins which are unable to activate the VirG protein.

Discussion

The VirA protein is an inner-membrane protein which acts as a receptor for plant phenolic compounds such as acetosyringone. To determine the role of the N-terminal part of the protein in the induction response, several hybrid proteins between VirA and the chemoreceptor Tar were constructed. For the protein Tar it is known that it exists as a homodimer and that the two transmembrane regions play an important role in dimerization and the maintenance of protein conformation [6, 16]. The periplasmic domain of the Tar protein is essential for ligand recognition and it is thought that binding of aspartate locks the dimer into a tighter complex, thereby decreasing the rate of dissociation and exchange of subunits in intact receptor dimers [21]. In this paper it is shown that the first 280 amino acids of the VirA protein are dispensable for the acetosyringone-mediated *vir* gene induction response, although exchanging this region by that of the Tar protein results in a hybrid protein which is weakly responsive to acetosyringone. This indicates that the conformation of the N-terminal part plays an important role in determining the efficiency of the *vir* gene induction response. Exchanging either one of the two transmembrane regions of VirA for those of Tar resulted in hybrid proteins which are inducible by acetosyringone, but non-responsive to monosaccharides such as D-glucose (Fig. 1A/B). This indicates that the two transmembrane regions are not merely hydrophobic membrane-

spanning regions, but rather play an important role in establishing the conformation of the periplasmic domain of the protein which is essential for the enhancement of the *vir* gene induction response by monosaccharides such as D-glucose. Whether the VirA protein also exists as a (homo)dimer, and whether amino acids located in the two transmembrane regions play a role in the process of dimerization (e.g. by forming a leucine zipper) is not known at the moment.

The region of the VirA protein acting as receptor domain for the phenolic compounds has not been determined yet. Turk *et al.* [30, 32] showed that the VirA proteins of different *Agrobacterium* strains, including the limited-host-range strains Ag57 and Ag162, are activated by acetosyringone. This indicates that the domain, acting as receptor for phenolic compounds must be conserved between the different VirA proteins. In an attempt to locate the region which is essential for the acetosyringone mediated *vir* gene induction response, Melchers *et al.* [19] showed that an active VirA protein which has an internal deletion of the periplasmic domain, the second transmembrane domain and part of the cytoplasmic linker region up to amino acid Arg-323 (pRAL7045) is still tumorigenic on *Nicotiana glauca* but non-responsive to acetosyringone. This suggests that a domain, important for the induction by the phenolic compounds is located within the deleted part of the protein. Results shown in Fig. 1 point out that the receptor domain for the phenolic compounds must be located in the cytoplasmic portion of the VirA protein since the N-terminal part of the VirA protein, including the second transmembrane domain, is not essential for the acetosyringone mediated *vir* gene induction. In order to determine the role of the cytoplasmic linker domain, near the second transmembrane domain, in the acetosyringone mediated *vir* gene induction response, several *virA* deletion mutants were constructed which encode mutant VirA proteins with deletions within this cytoplasmic region up to amino acid Arg-323 (Fig. 3). Table 2 shows that all deletions within the cytoplasmic linker region near the second transmembrane region of the VirA protein knock out the induction

			←
(pTiA6)	FLGSASVGLC	LYIITLVYRL	RKKTDWLARR
(pTi15955)			
(pRiA4)	..S.V.....	I...S.....	..R.....
(pTiC58)	V.....	I...S.....	..R..A..T..
(pTiAg162)	...AV..FF.	FG.VI..HK.	..RR..R....
pRAL7138	→←	pRAL7139	→
LDYEELIKEI	GVCFEGEAAT	----TSS---	-AQAALRIIQR
.....		-----	-.....
.....	GG..	----A....	-.....G....
....V....	GG..	-----LNS	S....V....
..F..V..K.	DSTE.	KQSLK.----	-E....GTIEN

Fig. 4. Comparison of the amino acid sequences of the linker region near the second transmembrane region from various VirA proteins. The sequence of pTiA6 is shown from amino acid 261 with the one-letter symbol. Identical amino acids are indicated by dots, missing amino acids are indicated by hyphens. The amino acids located in the second transmembrane region are indicated in bold. The regions deleted in pRAL7138 and pRAL7139, respectively, are indicated with arrows.

response. Since these deletions do not affect the kinase domain, it is likely that they affect the phenol-binding domain. However, it may equally well be that the deletions destroyed protein topology, thereby leading to mutants which are inactive or not targeted to the inner membrane of *Agrobacterium*. Acetosyringone is a very hydrophobic compound which accumulates in bacterial membranes (S. Turk, unpublished observations). Deleting part of the cytoplasmic linker region, between amino acids 283 and 323, affects the induction response by acetosyringone. Considering the fact that the cytoplasmic linker region between amino acids 283 and 304 is highly conserved between the different VirA proteins (Fig. 4) [5, 15, 18, 22], we suggest that the acetosyringone receptor domain is located in this cytoplasmic linker domain of the VirA protein.

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