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The Chimeric VirA-Tar Receptor Protein Is Locked into a Highly Responsive State

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The wild-type VirA protein is known to be responsive not only to phenolic compounds but also to sugars via the ChvE protein (G. A. Cangelosi, R. G. Ankenbauer, and E. W. Nester, Proc. Natl. Acad. Sci. USA 87:6708–6712, 1990, and N. Shimoda, A. Toyoda-Yamamoto, J. Nagamine, S. Usami, M. Katayama, Y. Sakagami, and Y. Machida, Proc. Natl. Acad. Sci. USA 87:6684–6688, 1990). It is shown here that the mutant VirA(Ser-44, Arg-45) protein and the chimeric VirA-Tar protein are no longer responsive to sugars and the ChvE protein. However, whereas the chimeric VirA-Tar protein was found to be locked in a highly responsive state, the VirA(Ser-44, Arg-45) mutant protein appeared to be locked in a low responsive state. This difference turned out to be important for tumorigenicity of the host strains in virulence assays on *Kalanchoë daigremontiana*.

The soil bacterium *Agrobacterium tumefaciens* is the causive agent of the plant disease crown gall and is capable of transferring part of its Ti plasmid DNA (T-DNA) to the plant cell nucleus. The expression of specific T-DNA genes results in the production of plant hormones, which cause cell proliferation and the formation of a tumor (6, 28, 30). The virulence genes *virA* and *virG* of the *A. tumefaciens* Ti plasmid are members of the two-component regulatory family and homologous to other members such as *envZ-ompR* and *dctB-dctD* (1, 21, 25). They enable the bacterium to sense plant phenolic compounds such as acetosyringone and mediate two distinct types of responses, i.e., chemotaxis at low concentrations of inducer (10^{-7} M) (19, 22) and induction of the *vir* genes at higher inducer concentrations ($>10^{-6}$ M). Chemotaxis enables the bacterium to migrate towards plant cells which are susceptible for transformation. *Vir* gene induction results in the production of proteins which are essential for the transfer of the T-DNA to the plant cell nucleus (24). The VirA protein is an innermembrane protein that is thought to act as a receptor for plant phenolic compounds such as acetosyringone (15, 16, 29). In vitro experiments have shown that the cytoplasmic portion of the VirA protein has autokinase activity and is able to phosphorylate VirG (7, 9, 10). The VirG protein binds to specific sequences in the *vir* promoters (2, 11, 20), and it is thought that the phosphorylated VirG protein is an efficient activator of *vir* gene transcription. Exactly how the phosphorylated VirG protein is able to regulate chemotaxis towards the phenolic inducers is not known at the moment.

Although it is still unclear whether the interaction between VirA and the phenolic inducer is direct or requires other phenolic binding proteins, it is known that the N-terminal part of the protein, including the periplasmic domain, is not essential for induction by phenolic compounds (3, 13, 15). Different regions in the periplasmic domain, however, are determinants of pH and temperature sensitivity of *vir* induction (15). Monosaccharides, such as D-glucose, play a synergistic effect in *vir* induction by phenolic compounds (3, 23). It is thought that the ChvE protein, which is homologous to the *Escherichia coli* galactose-binding protein, is able to bind monosaccharides and enhance *vir* induction, probably by binding to the periplasmic domain of VirA (3), since deleting part of this region results in a protein which is nonresponsive to monosaccharides such as D-glucose (3). Melchers et al. (15) reported that exchanging the periplasmic domain of VirA with that of the chemoreceptor Tar, a member of the class of methyl-accepting chemotaxis proteins and a receptor for aspartate, maltose, and phenol (4, 8), results in a hybrid protein which is less thermosensitive, less pH sensitive, and highly responsive to phenolic compounds. The last characteristic is surprising since this protein has an altered periplasmic domain, to which the ChvE protein probably is not able to bind anymore and, as a consequence, *vir* gene induction probably can no longer be enhanced by the presence of monosaccharides such as D-glucose.

In order to make a functional analysis of the VirA-Tar hybrid construct, several plasmids were constructed (Fig. 1) (15). Plasmid pST7111 was constructed by cloning a 4.5-kb *Eco*RI-*Bam*HI fragment of pRAL3254 (16), containing the complete *virA* gene of pTi15955, into *Eco*RI-*Bam*HI-digested pTZ18R (U.S. Biochemical Corporation, Cleveland, Ohio). This plasmid was mutagenized according to the method of Kunkel et al. (12), resulting in the cassette construct pST7116 in which three unique restriction sites are present and which is coding for the mutant VirA(Arg-15, Ser-44, Arg-45, Val-251) protein. Plasmid pST7117 was constructed by cloning a 0.5-kb *Nhe*I-*Hpa*I fragment of pRBB6 (15), coding for the periplasmic domain of the chemoreceptor Tar, into an *Xba*I-*Hpa*I-digested pST7116. These plasmids were linearized with *Eco*RI and cloned into the unique *Eco*RI site of the IncP class vector pRL750 (15), resulting in pRAL7111, pRAL7116, and pRAL7117. Cointegrates were transferred to *A. tumefaciens* LBA2524 and LBA2391 by electroporation (14).

As a control, first the effects that were caused by the introduction of the different restriction sites in the coding region of the *virA* gene were determined. Therefore, plasmid pRAL7111, a construct containing the wild-type *virA*, and

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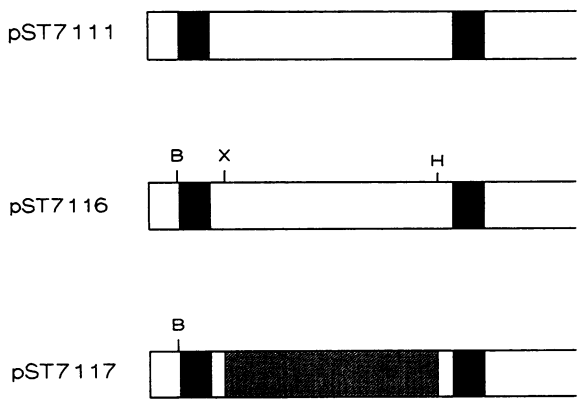

FIG. 1. Schematic representation of *virA* constructs. Black bars represent the coding region of the two transmembrane regions; the stippled bar indicates the coding region of the periplasmic domain of the chemoreceptor Tar. B, *Bss*H2; X, *Xba*I; H, *Hpa*I.

plasmid pRAL7116, a construct containing the *virA* gene with the restriction sites, were introduced into *A. tumefaciens* LBA2524(Δ*virA virB-lacZ*) (15) and tested for their ability to induce a *virB-lacZ* reporter construct. Figure 2 shows that in LBA2524(pRAL7111), acetosyringone-mediated *vir* gene expression is enhanced by the presence of 10 mM D-glucose, while this is not the case for LBA2524 (pRAL7116). In order to determine which restriction site was responsible for this defect, strains with *virA* constructs in which only one of the new restriction sites was introduced were similarly tested, as well as for their ability to provoke tumors on *Kalanchoë daigremontiana*, a plant species for which the enhancement of *vir* gene induction by sugars is essential for tumor formation (3). *Vir* gene induction experiments and tumor assays revealed that the mutant VirA(Ser-44, Arg-45) protein encoded by *virA*(*Xba*I) is nonresponsive to sugars, whereas VirA proteins encoded by genes in which an *Hpa*I or *Bss*H2 site had been introduced behaved like wild-type VirA (data not shown). The results indicate that either (i) the region containing Ser-44 and Arg-45 is important for the ChvE effect, although the proposed ChvE-binding site is located elsewhere in the periplasmic domain (3), or (ii) the positive charge, introduced by arg-45, changes protein conformation (18), thereby locking the receptor in a low responsive state.

Determining the *vir* induction characteristics of LBA2524 (pRAL7117), a strain containing the *virA-tar* hybrid construct, revealed that aspartate (1 mM) and phenol (200 μM), which are both inducers of the chemoreceptor Tar, were not able to induce the *vir* genes (results not shown), while in the presence of 2 μM acetosyringone, both in the presence and absence of 10 mM D-glucose, the *vir* genes are highly expressed (Fig. 3a). In contrast in strain LBA2524 (pRAL7111), containing the wild-type *virA* gene, high levels of *vir* gene expression are observed only when both 2 μM acetosyringone and 10 mM D-glucose are present in the induction medium (Fig. 3a). This could indicate either that the VirA-Tar hybrid protein is locked in a highly responsive state or that this protein has become inducible by the ChvE protein even in the absence of inducing sugars. In order to discriminate between these two possibilities, plasmids pRAL7111 and pRAL7117 were introduced into *A. tumefaciens* LBA2391 (*chvE*::Tn*5* Δ*virA virB-lacZ*) (27) and assayed for their capacity to mediate *vir* gene induction in the

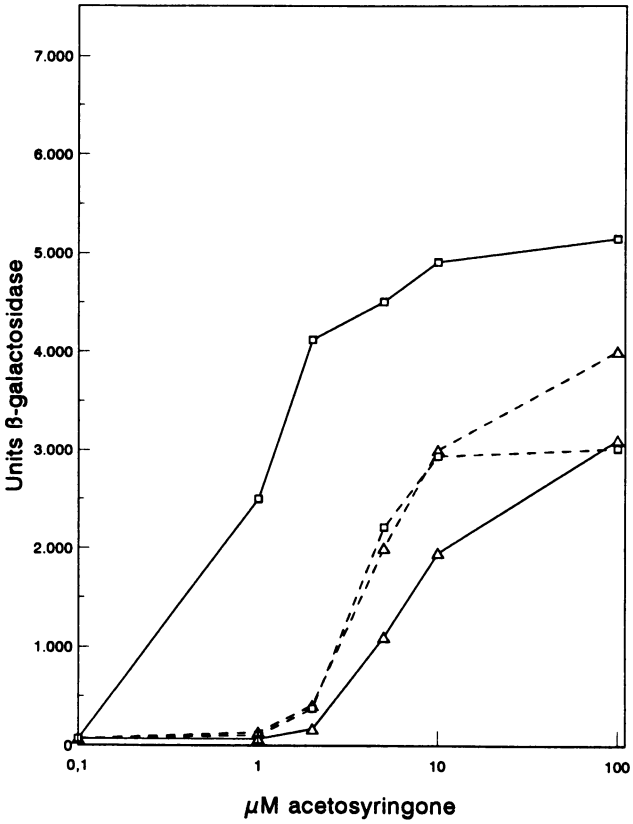

FIG. 2. Induction characteristics of the VirA(Arg-15, Ser-44, Arg-45, Val-251) protein. LBA2524 harboring pRAL7111 (——) or pRAL7116 (– – –) was grown overnight in minimal medium (5) supplemented with 0.5% (wt/vol) glycerol at 28°C and diluted 1:20 in induction medium (26) supplemented with 0.5% (wt/vol) glycerol and various concentrations of acetosyringone in the presence (□) or absence (△) of 10 mM glucose. Flasks were shaken for 18 h at 28°C, and β-galactosidase activity was determined as described by Miller (17).

absence of ChvE. The results shown in Fig. 3b indicate that the VirA-Tar protein mediates efficient *vir* induction in the absence of ChvE, in contrast to wild-type VirA. This indicates that the VirA-Tar hybrid protein is locked in a highly responsive state and thus is able to facilitate the production of high levels of *vir* gene products, independent of sugars and ChvE. Determining whether this ChvE independent *vir* gene induction of the VirA-Tar hybrid protein is reflected in a tumor assay, various strains were tested for their ability to provoke tumors on *K. daigremontiana*. Figure 4 shows that indeed strains which show only basal levels of *vir* induction in vitro even in the presence of D-glucose [i.e., LBA2391 (pRAL7111) and LBA2524(pRAL7116)] are avirulent on this plant species. However, in agreement with results from the in vitro *vir* induction experiments, a *chvE* mutant in which the gene for the VirA-Tar hybrid protein had been introduced had regained the ability to induce tumors on *K. daigremontiana*. The VirA-Tar hybrid protein should prove useful in further studies of the *vir* gene induction pathway, such as in determining the role of the periplasmic domain in chemotaxis towards acetosyringone (19) as well as in increasing the efficiency of transformation frequency of plant species in which phenolic inducers are limiting.

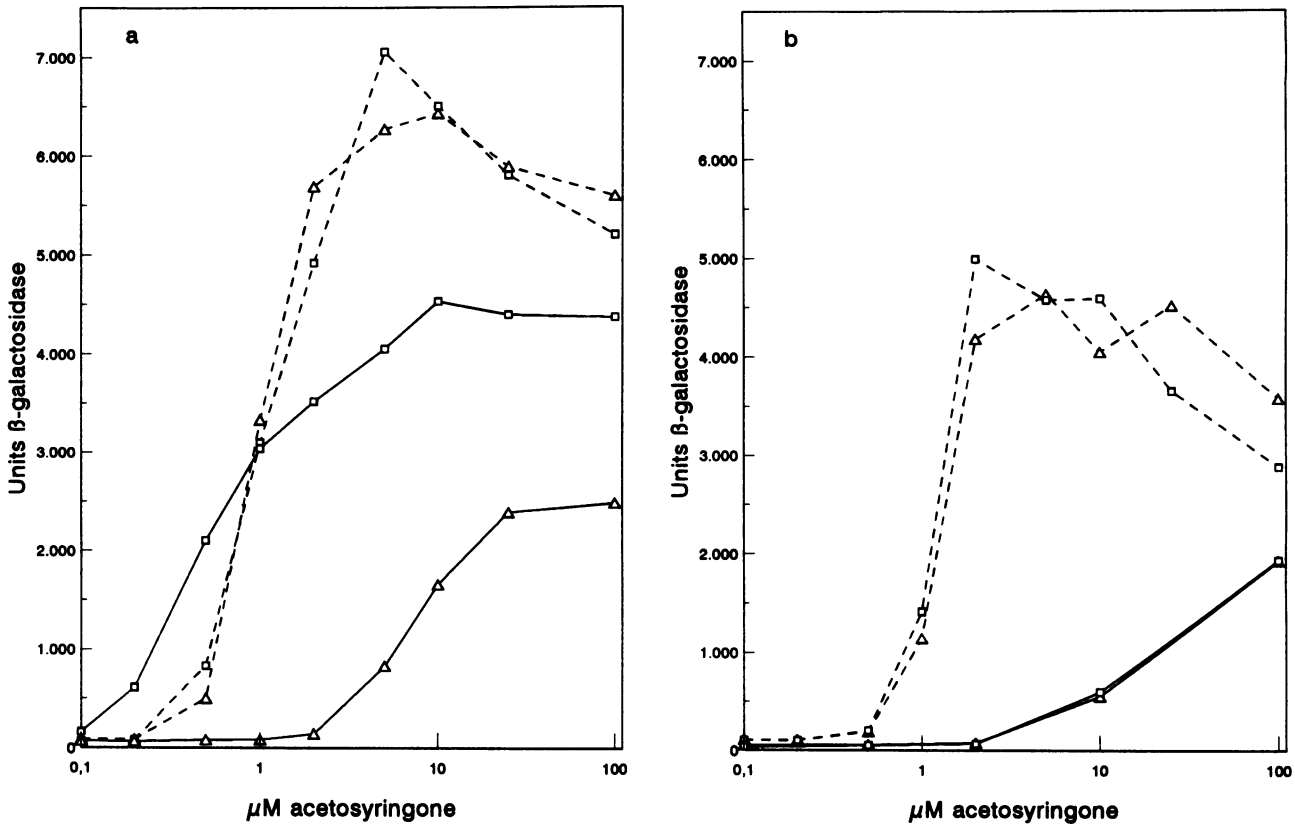

FIG. 3. Induction characteristics of the chimeric VirA-Tar protein. LBA2524 (a) or LBA2391 (b) harboring pRAL7111 (——) or pRAL7117 (– – –) was incubated in induction medium in the presence (□) or absence (△) of 10 mM D-glucose with the indicated concentration of acetosyringone. Flasks were shaken for 18 h at 28°C, and β-galactosidase activity was determined as described by Miller (17).

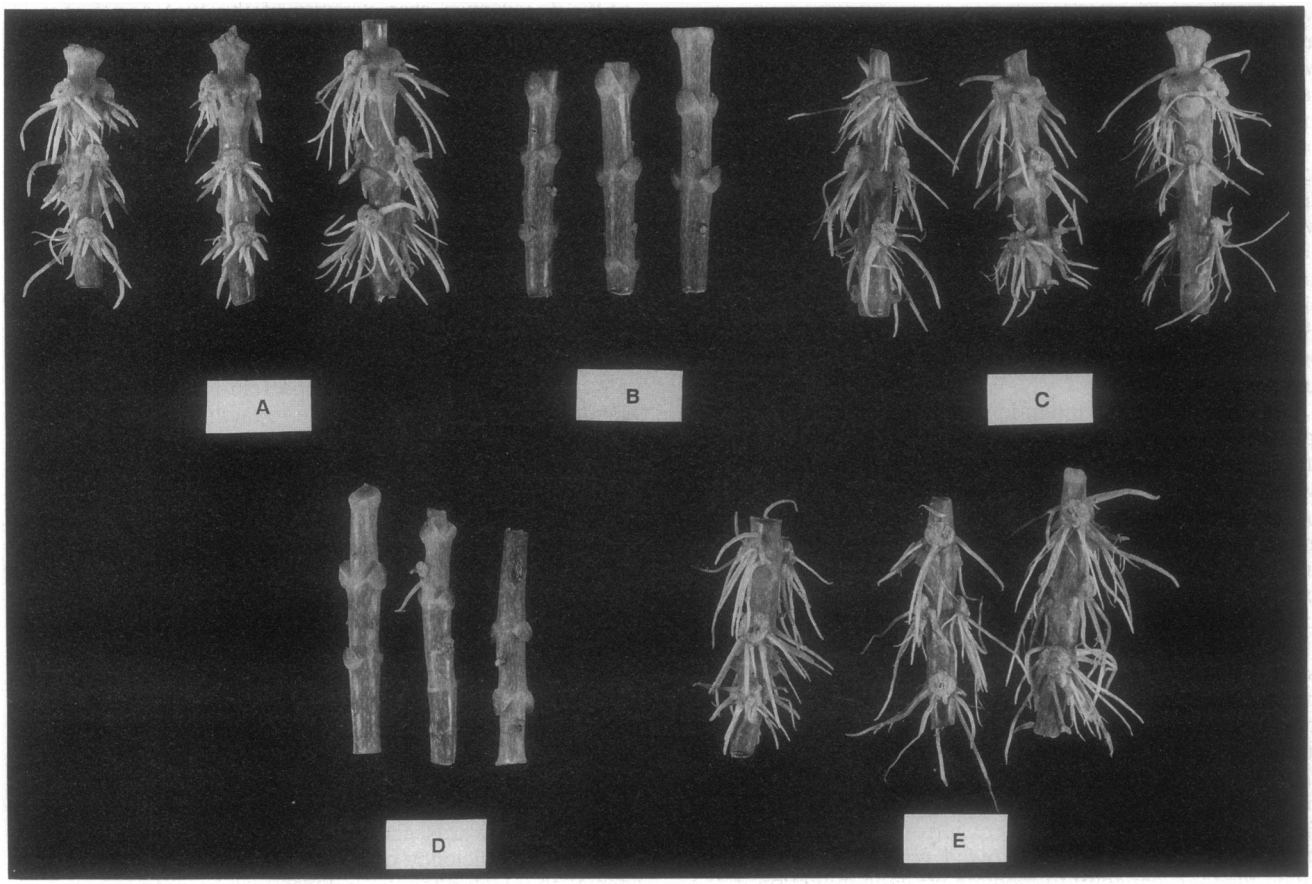

FIG. 4. Tumor induction on stems of *K. daigremontiana* upon infection with LBA2524 (A, B, and C) or LBA2391 (D and E) harboring pRAL7111 (A and D), pRAL7116 (B), or pRAL7117 (C and E). Tumor formation was evaluated after 3 weeks.

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