JOURNAL OF BACTERIOLOGY, Aug. 1998, p. 4300–4302 Vol. 180, No. 16
0021-9193/98/$04.00+0

Agrobacterium VirE2 Proteins Can Form a Complex with T Strands in the Plant Cytoplasm

STANTON B. GELVIN*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

Received 7 April 1998/Accepted 4 June 1998

Wild-type VirE2 and VirD2 proteins from *Agrobacterium tumefaciens* contain nuclear targeting sequences (NLS) that are likely involved in directing transferred T strands to the plant nucleus. An *A. tumefaciens virE2 virD2*ΔNLS double mutant was able to form tumors on VirE2-producing transgenic tobacco but not on wild-type tobacco. Because this mutant bacterial strain contains no known T-strand nuclear targeting signal, the data indicate that wild-type VirE2 proteins produced by the plant can interact with the T strands in the plant cytoplasm and direct them to the nucleus.

Agrobacterium tumefaciens incites crown gall tumors by processing a region of DNA (the transferred DNA, or T-DNA) from the resident Ti (tumor-inducing) plasmid and transferring a single-stranded form of this DNA (the T strand [27, 33]) to a plant cell (for recent reviews, see references 23, 35, and 36). To transform a plant cell, the T-DNA must target the nucleus and stably integrate into the plant genome. Nuclear targeting is thought to be mediated by nuclear localization sequences (NLS) found in two virulence (Vir) proteins likely to associate with the T-DNA in the plant cell. These proteins are VirD2, a single molecule of which is covalently linked to the 5′ end of the T strand (8, 12, 15, 30, 32), and VirE2, a non-sequence-specific single-stranded DNA binding protein that may noncovalently associate along the length of the T strand and protect it from nuclease degradation (2, 3, 5, 7, 11, 22). The abundance of VirE2 protein found in *A. tumefaciens* cells incubated with certain *vir* gene-inducing phenolic molecules (9), as well as the high affinity of this protein for single-stranded DNA (5), have led to the hypothesis that T-DNA is exported from *A. tumefaciens* as a VirE2-VirD2-nucleic acid complex called the "T-complex" (14). However, researchers have yet to identify this complex in *A. tumefaciens*.

Recently, results from several laboratories have prompted a reevaluation of the T-complex model as originally proposed. Many of these data derive from "extracellular complementation" experiments. Otten et al. (20) showed that two mutant *A. tumefaciens* strains, each separately avirulent, were able to incite tumors when coinoculated onto plants. These two strains were termed the VirE2 donor strain (containing a wild-type *virE2* gene but lacking T-DNA) and the T-DNA donor strain (containing T-DNA but lacking a functional *virE2* gene). Extracellular complementation implies that T-DNA and VirE2 protein can be exported separately into the plant and that the T strand does not have to be coated with VirE2 protein in the bacterium. Indeed, Yusibov et al. (33) showed that a *virE2* mutant *A. tumefaciens* strain was able to transfer T-DNA to the cytoplasm of tobacco cells, although the steady-state amount of T-DNA found in the plant cells was less than that found upon infection with a wild-type *A. tumefaciens* strain. More recently, Binns et al. (1) showed that the oncogenic suppressive IncQ plasmid RSF1010 preferentially inhibited the VirE2 donor strain, rather than the T-DNA donor strain, in extracellular complementation experiments. Sundberg et al. (26), using a similar experimental protocol, showed that VirE1 protein was required for VirE2 protein export, but not T-DNA export, from *A. tumefaciens*. Finally, Lee et al. (18) showed that the oncogenic suppressive IncW plasmid pSa inhibited VirE2 protein but not T-DNA export from the bacterium. Taken together, these data suggest that VirE2 protein and T-DNA are separately transferred from *A. tumefaciens* to plant cells.

If T-DNA and VirE2 protein were separately transported to the plant cell, and if VirE2 protein were important both for protection of the T-DNA from plant nucleases (22, 33) and for targeting the T-DNA to the nucleus (4, 6, 34), then VirE2 protein must be able to form a complex with T-DNA in the plant cell. Citovsky et al. (6) showed that in transgenic tobacco expressing a GUS-VirE2 fusion protein, all detectable GUS activity was localized to the nucleus. They also showed that a VirE2-producing transgenic tobacco plant could be stably transformed by a *virE2* mutant *A. tumefaciens* strain, whereas a wild-type plant could not. These data imply that VirE2 protein can form a complex with the T strand in the plant cell. However, it is not clear from the work of Citovsky et al. (6) whether VirE2 protein interacted with the T strand before or after entry of the T-DNA into the nucleus. This is because the *A. tumefaciens* strain used in these experiments contains a wild-type *virD2* gene that encodes a protein that itself contains an NLS (13, 16, 21, 24, 28). Either VirD2 protein was responsible for nuclear targeting of the T-DNA, or a small, undetectable amount of VirE2 protein remained in the cytoplasm and complexed with the T strand before nuclear entry. I therefore have tested the hypothesis that VirE2 protein, when expressed in a plant cell, can direct the T strand to the nucleus in the absence of an NLS from any other known T-DNA-associated virulence protein.

VirD2 protein contains two NLS regions that, when individually fused to a reporter protein, can direct reporter protein activity to the plant nucleus (13, 16, 21, 24, 28). However, several laboratories have shown that the N-terminal monopartite NLS is not involved in targeting T strands to the nucleus (17, 21, 24). This is because the T strand that attaches to tyrosine-29 of VirD2 protein (29) likely occludes this N-terminal NLS. Only the C-terminal bipartite NLS is able to target the T-DNA to the nucleus. Using linker scanning and deletion mutations in the C-terminal region of *virD2*, Shurvinton et al.

* Mailing address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392. Phone: (765) 494-4939. Fax: (765) 496-1496. E-mail: gelvin@bilbo.bio.purdue.edu.

TABLE 1. Tumorigenesis of *A. tumefaciens* wild-type and mutant strains on normal and VirE2 transgenic tobacco leaf discs[a]

A. tumefaciens strain	Strain description	Tobacco plant	No. of discs infected	No. (%) of discs with:			
				0 tumors	1 tumor	2 tumors	≥3 tumors
A136	No pTi	Wild-type	15	15 (100)	0 (0)	0 (0)	0 (0)
		VirE2	19	19 (100)	0 (0)	0 (0)	0 (0)
A348	Wild-type pTiA6	Wild-type	18	4 (22)	2 (11)	3 (17)	9 (50)
		VirE2	22	3 (14)	3 (14)	3 (14)	13 (59)
At54	*virE2*::Tn*3*-HoHo1	Wild-type	19	19 (100)	0 (0)	0 (0)	0 (0)
		VirE2	21	6 (28)	1 (5)	3 (14)	11 (52)
WR1766	*virD2*ΔNLS	Wild-type	25	1 (4)	5 (20)	2 (8)	17 (68)
		VirE2	22	8 (36)	6 (27)	0 (0)	8 (36)
At1015	*virE2*::Tn*3*-HoHo1 *virD2*ΔNLS	Wild-type	24	24 (100)	0 (0)	0 (0)	0 (0)
		VirE2	24	9 (38)	4 (17)	3 (12)	8 (33)

[a] Discs were infected with *A. tumefaciens* strains at 10^9 cells/ml, and the numbers of tumors were scored 30 days later.

(24) showed that the bipartite NLS amino acids KRPR and RKRAR are the only regions involved in nuclear targeting. Furthermore, Mysore et al. (19) showed that when the amino acids delimited by the KRPR and RKRAR motifs were altered but the overall charge of the region was maintained, the remaining amino acids were unable to target a VirD2-GUS fusion protein to the nucleus. In the following experiment, I therefore assumed that the KRPR and RKRAR motifs are the only sequences of VirD2 protein involved in targeting the T strand to the nucleus.

I separately infected wild-type and VirE2-producing transgenic tobacco (6) leaf discs with five different *A. tumefaciens* strains and recorded the number of tumors per disc 30 days later. In Table 1, the percentage of virulence was calculated by comparing the number of discs with at least one tumor to the total number of discs infected. *A. tumefaciens* A136 (31) lacks a Ti plasmid and is therefore avirulent. *A. tumefaciens* A348 (10) contains the octopine-type Ti plasmid pTiA6 with a wild-type *virD2* gene. *A. tumefaciens* At54 (A348mx361 [25]) contains the transposon Tn*3*-HoHo1 in the *virE2* gene. This strain is avirulent on tobacco. *A. tumefaciens* WR1766 (24) contains a nonpolar deletion of most of *virD2* in strain WR5000 and plasmid pWR1766, containing a mutant *virD2* gene in which the NLS amino acids KRPR and KRAR have been deleted (*virD2*ΔNLS). This strain maintains approximately 60% virulence on potato tuber discs (24) and almost full virulence on tobacco (19). I constructed the double mutant *A. tumefaciens* At1015 (*virE2 virD2*ΔNLS) as follows. I introduced the *virE2*::Tn*3*-HoHo1 mutation from pSM361 (25) into the Ti plasmid of *A. tumefaciens* WR5000 by marker exchange mutagenesis (10). I subsequently introduced into this mutant strain plasmid pWR1766. This double-mutant strain is avirulent on tobacco (Table 1).

Table 1 shows that, as previously shown by Citovsky et al. (6), the *virE2* mutant *A. tumefaciens* At54 cannot incite tumors on wild-type tobacco but can on VirE2-producing transgenic tobacco. In this experiment, At54 showed approximately half the virulence of the wild-type strain A348 on VirE2-producing transgenic plants. The *virD2*ΔNLS mutant WR1766 showed high virulence (96%) on wild-type tobacco and slightly reduced virulence (64%) on VirE2-producing transgenic tobacco. Most importantly, the *virE2 virD2*ΔNLS double mutant At1015 showed a high level of virulence (62%) on VirE2-producing transgenic tobacco. The transformation experiments were repeated with similar results (data not shown).

The VirD2ΔNLS–T-DNA complex of *A. tumefaciens* At1015 contains no known nuclear targeting signal. The results reported in Table 1 indicate that VirE2 protein must be able to associate with this complex in the plant cytoplasm and direct it to the nucleus. Zupan et al. (34) showed that VirE2 protein, when complexed in vitro with single-stranded DNA, can direct the DNA to the nucleus when microinjected into plant cells. My results further indicate that although VirE2 protein can direct itself and previously associated nucleic acids to the nucleus, it can also find T strands introduced into the plant cytoplasm by *A. tumefaciens* and target them to the nucleus. Although not proof, this finding is consistent with the hypothesis that *A. tumefaciens* separately transfers VirE2 protein and a VirD2–T-DNA complex to the plant.

I thank Vitaly Citovsky for supplying seeds of VirE2-producing transgenic tobacco plants; Walt Ream for supplying the *A. tumefaciens* strains WR5000 and WR1755, for useful discussions, and for critical review of the manuscript; and Lan-Ying Lee for help with the leaf disc transformations and for critical reading of the manuscript.

This work was supported by a grant from the U.S. Department of Agriculture (95-37301-2040).

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