

Delineation of the Interaction Domains of *Agrobacterium tumefaciens* VirB7 and VirB9 by Use of the Yeast Two-Hybrid Assay

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The *Agrobacterium tumefaciens* VirB proteins are postulated to form a transport pore for the transfer of T-DNA. Formation of the transport pore will involve interactions among the VirB proteins. A powerful genetic method to study protein-protein interaction is the yeast two-hybrid assay. To test whether this method can be used to study interactions among the VirB membrane proteins, we studied the interaction of VirB7 and VirB9 in yeast. We recently demonstrated that VirB7 and VirB9 form a protein complex linked by a disulfide bond between cysteine 24 of VirB7 and cysteine 262 of VirB9 (L. Anderson, A. Hertz, and A. Das, Proc. Natl. Acad. Sci. USA 93:8889-8894, 1996). We now demonstrate that VirB7 and VirB9 interact in yeast, and this interaction does not require the cysteine residues essential for the disulfide linkage. By using defined segments in fusion constructions, we mapped the VirB7 interaction domain of VirB9 to residues 173 to 275. In tumor formation assays, both *virB7C24S* and *virB9C262S* expressed from a multicopy plasmid complemented the respective deletion mutation, indicating that the cysteine residues may not be essential for DNA transfer.

Agrobacterium tumefaciens transfers a segment of its tumor-inducing (Ti) plasmid DNA upon infection of plant cells (reviewed in reference 15). Expression of the phytohormone biosynthetic genes encoded within the transferred DNA leads to the crown gall tumor phenotype. The transfer of the T-DNA is catalyzed by proteins encoded in the virulence (*vir*) region of the Ti plasmid (12, 20, 31). Proteins of the *virB* operon and one protein encoded in the *virD* operon, VirD4, are essential for DNA transfer (42). These proteins also catalyze the transfer of plasmid DNA between two *Agrobacterium* strains and from *Agrobacterium* to the yeast *Saccharomyces cerevisiae* (2, 5, 22, 26).

The *virB* operon encodes 11 proteins, 10 of which, VirB2 to VirB11, are essential for DNA transfer (4, 38). The other one, VirB1, augments transfer efficiency. The presence of one or more hydrophobic segments that can function in membrane spanning and/or as a signal sequence in most VirB proteins suggests that these proteins are membrane proteins or are associated with the bacterial membranes (17, 33, 38). This hypothesis is supported by biochemical studies that demonstrated that all 11 VirB proteins fractionate with the bacterial membrane (9, 11, 28, 34). Mutational studies demonstrated that *virB* is not required for the synthesis of T-strand DNA, the intermediate in T-DNA transfer (32, 35, 43). The association of the VirB proteins with the bacterial membranes, the essential role of these proteins in DNA transfer, and their requirement in a step(s) beyond the T-strand DNA synthesis led to the hypothesis that the VirB proteins form a transport pore to allow T-strand DNA to move from bacteria to a plant cell. The observations that the transfer proteins of the bacterial conjugal plasmids and the Ptl proteins of the animal pathogen *Bordetella*

pertussis are homologous to the VirB proteins support this hypothesis (18, 23, 40). The transfer proteins are essential for the conjugal transfer of the plasmid DNA, and the Ptl proteins are essential for the excretion of a toxin protein.

The transport pore for T-DNA transfer is probably a complex structure involving several proteins. Protein-protein interactions are likely to play a major role in the assembly and stabilization of this structure. We recently reported the identification of the first protein complex composed of two VirB proteins (1). We demonstrated that VirB7 and VirB9 form a covalent complex linked by a disulfide bond. Cysteine 24 of VirB7 and cysteine 262 of VirB9 participate in the disulfide bond formation. Similar results were obtained by Spudich et al. (30). Since VirB7 is a lipoprotein and is anchored to the outer membrane (8), it is postulated that the VirB7-VirB9 complex is linked to the inner surface of the outer membrane. In an earlier study, a homooligomer of VirB10 was identified by use of a chemical cross-linker (39).

Because protein-protein interactions probably play a major role in the organization of the transport pore, the identification of the proteins involved in these interactions is essential to define the assembly and architecture of the pore. A genetic method to study such interactions is the yeast two-hybrid assay (10). In the last several years, this system has been widely used to identify interacting proteins (reviewed in reference 41). Almost all proteins used in these studies are of cytoplasmic origin. Since all VirB proteins are membrane proteins and very limited information is available on whether the two-hybrid assay can be used to study membrane proteins, we used the VirB7-VirB9 system as a test system to investigate the suitability of this approach to study interactions among the VirB proteins. Since the existence of the VirB7-VirB9 complex has been established by biochemical methods, we considered that this complex will be a good experimental system for analysis by the yeast two-hybrid assay. In this study, we demonstrate that VirB7 and VirB9 interact with each other in a yeast two-hybrid assay and define the sequences essential for this interaction.

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TABLE 1. Strains and plasmids

| Strain or plasmid | Relevant characteristics ^a | Source or reference |
|-----------------------------|---|---------------------|
| Strains | | |
| <i>E. coli</i> DH5 α | <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA relA1</i> λ^- ϕ 80d/ <i>lacZ</i> Δ M15(Δ <i>lacZYA-argF</i>) <i>U169</i> | Laboratory stock |
| <i>A. tumefaciens</i> | | |
| A136 | C58 heat cured of pTiC58 | Laboratory stock |
| A348 | A136 containing pTiA6 | Laboratory stock |
| A348 Δ B7 | A348 containing a deletion in pTiA6 <i>virB7</i> | 4 |
| A348 Δ B9 | A348 containing a deletion in pTiA6 <i>virB9</i> | 4 |
| <i>S. cerevisiae</i> | | |
| EGY48 | <i>ura3 his3 trp1 LexAop-leu2</i> | 13 |
| AD842 | EGY48 containing pSH18-34 | This study |
| Plasmids | | |
| pEG202 | Cloning vector for the construction of LexA fusions | 13 |
| pJG4-5 | Cloning vector for the construction of activator fusions | 13 |
| pSH18-34 | A plasmid containing <i>Gal1-LexA_{op}-lacZ</i> , a reporter gene in yeast | 13 |
| pBSKSII(+) | Cloning vector | Stratagene |
| pBSKSGnII(+) | A gentamicin-resistant derivative of plasmid pBSKSII(+) | F. White |
| pTJS75 | Cloning vector; a WHR IncP plasmid | 27 |
| 75 Δ 2 | A deletion mutant of pTJS75 | 6 |
| pAD1378 | A 7.5-kb <i>EcoRI</i> fragment containing plasmid 75 Δ 2 and <i>virGN54D</i> in a chloramphenicol-resistant derivative of pUC118 | 1; this study |
| pVK222 | Source of pTiA6 <i>virB</i> | 16 |
| pAD1287 | A 10.5-kb <i>NdeI-XhoI</i> fragment (residues 109 to 10567 of reference 38) encoding pTiA6 <i>virB</i> in pBSKSII(+) | 1 |
| pAV134R | A 5.3-kb <i>SacI</i> fragment of pAD1287 containing <i>virB6-virB11</i> in pBSKSGnII(+) | 1 |
| pAD1391 | A 2.9-kb <i>EcoRI</i> fragment containing <i>virB7-virB10</i> recloned in pAV134R digested with <i>EcoRI</i> | 1 |
| pAD1416 | A 0.39-kb <i>virD</i> promoter fragment (-384 to +7) in pUC118 | This study |
| pAD1446 | A 0.87-kb <i>NaeI</i> fragment from pAD1391 in pEG202 (<i>lexA-virB9</i> fusion) | This study |
| pAD1494 | A 0.9-kb <i>EcoRI-XhoI</i> fragment of pAD1446 in pJG4-5 (activator- <i>virB9</i> fusion) | This study |
| pAD1487 | Same as pAD1446 except that it contains <i>virB9C262S</i> | This study |
| pAD1447 | A 0.25-kb <i>EcoRI</i> fragment containing <i>virB7</i> obtained by PCR amplification of pAD1391 in pJG4-5 (activator- <i>virB7</i> fusion) | This study |
| pAD1486 | Same as pAD1447 but contains <i>virB7C24S</i> | This study |
| pAD1483 | A 0.25-kb <i>EcoRI</i> fragment of pAD1447 in pEG202 (<i>lexA-virB7</i> fusion) | This study |
| pAD1389 | A WHR <i>virB9</i> expression plasmid; contains <i>virD_p-virB9</i> and <i>virGN54D</i> ; pAD1388 in the <i>EcoRI</i> site of pAD1378; <i>Chl</i> ^s | 1 |
| pAD1399 | A <i>virD_p-virB9-virB7</i> chimeric operon in pUC118 | 1 |
| pAD1400 | A WHR <i>virB7</i> and <i>virB9</i> expression plasmid; pAD1399 in pAD1378; <i>Chl</i> ^s | 1 |
| pAD1423 | A <i>virD_p-virB7-virB8</i> chimeric operon in pUC118; a 1.06-kb <i>AlwNI-SphI</i> fragment of pAV134R in pAD1416 | This study |
| pAD1423C15S | Same as pAD1423 but contains <i>virB7C15S</i> | This study |
| pAD1423C24S | Same as pAD1423 but contains <i>virB7C24S</i> | This study |
| pAD1424 | pAD1423 in the <i>KpnI</i> site of pAD1378; <i>Chl</i> ^s | This study |
| pLA13 | A <i>virD_p-virB7-virB10</i> chimeric operon in pUC118; a 1.9-kb <i>SphI-HindIII</i> fragment of pAD1391 in pAD1423 digested with the same enzymes | This study |
| pLA20 | Deletion of <i>virB10</i> from pLA13 by <i>NsiI-HindIII</i> blunt self-ligation | This study |
| pLA21 | pLA20 in the <i>SacI</i> site of pAD1378; <i>Chl</i> ^s | This study |
| pLA23 | Same as pLA20 but contains <i>virB7C15S</i> ; an ~1.0-kb <i>BsrEII-HindIII</i> fragment of pLA20 in pAD1423C15S | This study |
| pLA24 | Same as pLA20 but contains <i>virB7C24S</i> ; an ~1.0-kb <i>BsrEII-HindIII</i> fragment of pLA20 in pAD1423C24S | This study |
| pLA26 | pLA23 in the <i>KpnI</i> site of pAD1378; <i>Chl</i> ^s | This study |
| pLA27 | pLA24 in the <i>KpnI</i> site of pAD1378; <i>Chl</i> ^s | This study |

^a WHR, wide-host-range; *Chl*^s, chloramphenicol sensitive.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. For the yeast two-hybrid assays, plasmids pEG202 and pJG4-5 were used for the construction of the bait and activator fusions (14). Plasmid pAD1446, a *lexA-virB9* fusion plasmid, was constructed by cloning a 870-bp *NaeI* fragment that encodes the C-terminal 267 residues of VirB9 into a filled-in *BamHI* site of plasmid pEG202. The fusion protein lacks the first 27 residues of VirB9. Plasmid pAD1447, an activator-*virB7* fusion plasmid, and plasmid pAD1483, a *lexA-virB7* fusion plasmid, were constructed by cloning a ~250-bp *EcoRI* fragment encoding residues 11 to 55 of VirB7 into the unique *EcoRI* site of pJG4-5 and pEG202, respectively. The fragment was generated by PCR

amplification of pAD1399 with a *virB7*-specific synthetic oligonucleotide and the m13/pUC reverse sequencing primer as the primers followed by digestion with *EcoRI* (24). Plasmids pAD1486 and pAD1487 are similar to pAD1447 and pAD1446 except that they contain *virB7C24S* and *virB9C262S*, respectively. Plasmid pAD1494, an activator-*virB9* fusion plasmid, was constructed by cloning the ~0.9-kb *EcoRI-XhoI* fragment of pAD1446 into plasmid pJG4-5. Derivatives of plasmid pAD1494 that contained defined sequences from the C-terminal end of VirB9 were constructed by cloning the appropriate fragments generated by PCR amplification (24). The junction regions of all fusion plasmids were confirmed by DNA sequence analysis (6, 25).

For the expression of the *virB* genes, the coding sequence of the gene(s) of

interest was placed under the control of the *virD* promoter (*virD_p*). The chimeric gene was cloned into a high-copy plasmid containing the mutant IncP plasmid 75Δ2 (6) and the *vir* gene constitutive mutant *virGN54D* (21). Plasmid pAD1424 contains *virB7* and *virB8*. Plasmids pLA21, pLA26, and pLA27 contain both *virB8* and *virB9* plus either *virB7*, *virB7C15S*, or *virB7C24S*, respectively.

Two-hybrid assay. Plasmid DNA was introduced into yeast by transformation (13). Briefly, a fresh colony grown on a yeast extract-peptone-dextrose plate was resuspended in 30 μl of 0.1 M lithium acetate. Nine volumes of a solution containing 0.1 M lithium acetate, 45% polyethylene glycol-3350, 1 mM EDTA, and 10 mM Tris-HCl (pH 8) was added. DNA (1 to 5 μg) was added to the cell suspension, mixed by vortexing, and incubated overnight at room temperature. The next day, cells were plated on selective medium.

For two-hybrid assays, we constructed a yeast strain, AD842, by introducing the plasmid pSH18-34 that contains a reporter *Gall*-LexAop-*lacZ* gene into *S. cerevisiae* EGY48 by transformation. Derivatives of the bait and the activator plasmids containing the appropriate gene fusions were then introduced into *S. cerevisiae* AD842. For control experiments, the vector plasmid was introduced into the strain containing a fusion gene. The interaction between two proteins was tested by assaying for a blue colony on plates containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and by growth on a leucine-free medium. The host strain contains a *Gall*-LexAop-*leu2* gene on the chromosome. Activation of *Gall* results in a *leu⁺* phenotype.

For β-galactosidase assays, cells were grown overnight in the presence of galactose and raffinose in complete minimal dropout medium lacking uracil, tryptophan, and histidine (13). Cells were diluted to an *A*₆₀₀ of ~0.2 and grown overnight in the same medium. β-Galactosidase activity was assayed as described by Miller (19). The data presented are averages of two independent experiments.

Tumorigenesis assays. The ability of *virB7*, *virB9*, and their mutants to complement a deletion in the respective gene was investigated by monitoring tumor formation on *Kalanchoe daigremontiana* leaves and potato tubers as described previously (29, 37).

RESULTS AND DISCUSSION

A two-hybrid system to study interactions among the VirB proteins. The *vir*-mediated DNA transfer of T-DNA from *Agrobacterium* to plant cells is postulated to occur through a transport pore composed primarily of the VirB proteins. An assembly of the transport pore will require a specific interaction between the VirB proteins. We recently reported that two VirB proteins, VirB7 and VirB9, form a disulfide-linked protein complex (1). Cysteine 24 of VirB7 and cysteine 262 of VirB9 participate in the disulfide bond formation. A powerful genetic method to identify proteins that interact with each other is the two-hybrid assay in the yeast *S. cerevisiae* (10). This system has been used successfully to study soluble proteins. We investigated whether this system can be used to define protein-protein interactions among the VirB membrane proteins. As a test case, we studied the interaction between VirB7 and VirB9.

The system developed by R. Brent and coworkers was used for the two-hybrid assays (13, 14). The coding region of VirB9 except for that encoding the first 27 residues was cloned into plasmid pEG202 to construct a *lexA-virB9* gene fusion, the bait gene. An activator-*virB7* fusion was constructed by cloning a DNA fragment encoding the C-terminal 45 residues of VirB7 (residue 11 to the end). To test whether the VirB7 and VirB9 fusion proteins interact with each other, plasmids containing the appropriate fusions were introduced into a yeast strain harboring the reporter genes *Gall*-LexAop-*leu2* and *Gall*-LexAop-*lacZ*. A strain expressing the LexA-VirB9 fusion and the activator or the activator-VirB7 fusion and LexA did not grow in the absence of leucine and formed white colonies on indicator plates containing X-Gal (Fig. 1). When both the LexA-VirB9 and the activator-VirB7 fusions were expressed in the same host, the recombinant grew in a leucine-free medium and formed blue colonies on the indicator plate. Leucine prototrophy and the formation of a blue colony require activation of the *Gall* promoter, and *Gall* activation requires that the DNA binding and activator domains of the two fusion proteins are positioned together on the DNA. A positive result in these studies indicates that the VirB7 and VirB9 segments of the fusion proteins bring the two functional domains together by

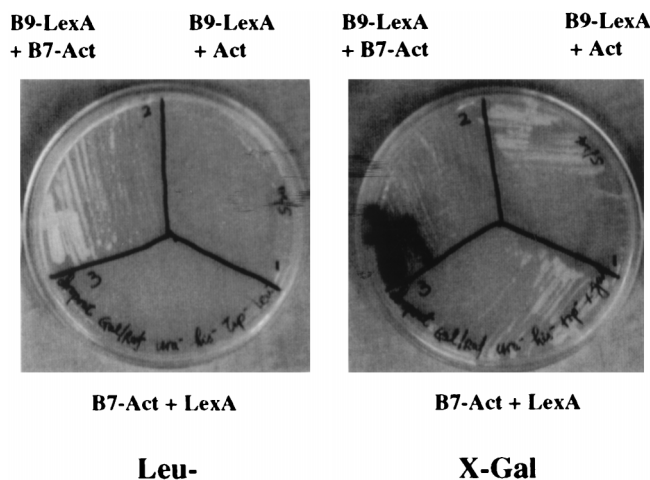


FIG. 1. Protein-protein interaction between VirB7 and VirB9. Interaction between VirB7 and VirB9 was monitored by the yeast two-hybrid assay (13). A yeast strain containing a plasmid(s) that expresses the desired fusion protein was streaked on a plate lacking leucine (Leu⁻) and on one containing X-Gal. The restoration of *Gall* transcription by interaction between two fusion proteins allows expression of a *leu2* gene and a reporter *lacZ* gene. The plasmid contents of the three strains studied are indicated on the figure. LexA, LexA vector plasmid; Act, activator vector plasmid; B9-LexA, LexA-VirB9 fusion; B7-Act, activator-VirB7 fusion. All strains harbor another plasmid containing the *Gall*-LexAop-*lacZ* gene.

protein-protein interactions. These results confirm our earlier observations that VirB7 and VirB9 interact with each other.

Role of the cysteine residues essential for disulfide bond formation in the VirB7-VirB9 interaction. VirB7 and VirB9 form a covalent complex linked by a disulfide bond between cysteine 24 of VirB7 and cysteine 262 of VirB9 (1, 30). The alteration of the cysteines to serines led to the loss of the disulfide-linked complex; however, what effect, if any, the mutations have on protein function was not assessed in those studies. The success of the two-hybrid assay suggests that the cysteine residues are probably not essential for the VirB7-VirB9 complex formation because the reducing environment of a yeast nucleus is unlikely to support disulfide bond formation. To test this hypothesis, we constructed fusions that contain cysteine-to-serine substitutions in VirB7 and VirB9 and studied the effect of the mutations on reporter gene expression by the two-hybrid assay (Table 2). A yeast strain expressing LexA-VirB9C262S and activator-VirB7 formed blue colonies

TABLE 2. Role of VirB7 cysteine 24 and VirB9 cysteine 262 in VirB7-VirB9 interaction^a

| LexA fusion | Activator fusion | β-Galactosidase activity (Miller units) ^b |
|-------------|------------------|--|
| VirB9 | VirB7 | 14 ± 0.2 |
| VirB9 | VirB7 | 7 ± 0.1 |
| VirB9C262S | VirB7 | 233 ± 3 |
| VirB9C262S | VirB7 | 19 ± 0.5 |
| VirB9C262S | VirB7C24S | 291 ± 40 |
| VirB9 | VirB7C24S | 11 ± 0.4 |
| VirB9 | VirB7C24S | 146 ± 8 |
| VirB9C262S | VirB7C24S | 273 ± 3 |

^a Interactions between VirB7, VirB9, and their mutants were monitored by the yeast two-hybrid assay (13). Expression of the reporter *lacZ* gene was measured by the method of Miller (19).

^b Data presented are averages of two independent experiments ± standard errors.

TABLE 3. Identification of the VirB9 domain that interacts with VirB7

| LexA fusion | VirB9 residues in the activator fusion | β -Galactosidase activity (Miller units) ^a |
|-------------|--|---|
| VirB7 | 28–293 | 18 \pm 0.5 |
| | 28–293 | 1,090 \pm 30 |
| | 173–293 | 9 \pm 0.5 |
| VirB7 | 173–293 | 2,011 \pm 60 |
| | 226–293 | 7.5 \pm 0.2 |
| VirB7 | 226–293 | 9 \pm 0.6 |
| | 173–275 | 2 |
| VirB7 | 173–275 | 2,741 \pm 160 |
| VirB7 | 226–275 | 7 \pm 0.2 |
| VirB7 | 214–275 | 5.5 \pm 0.5 |
| VirB7 | 197–275 | 3.5 \pm 0.6 |

^a Values are means \pm standard errors.

on a plate containing X-Gal (data not shown) and showed a high level of β -galactosidase activity. Similarly, strains expressing LexA-VirB9 and activator-VirB7C24S had a *lac*-positive phenotype. A strain that expressed both VirB7C24S and VirB9C262S had a high level of β -galactosidase activity, a level comparable to that in a strain expressing both of the wild-type proteins. These results indicate that the cysteine residues that participate in disulfide bond formation are not required for VirB7-VirB9 interaction in yeast.

A 103-residue domain near the C terminus of VirB9 is sufficient for complex formation with VirB7. The yeast two-hybrid system was used to more precisely define the VirB7 interaction domain of VirB9. For our initial analysis, we constructed fusions of VirB9 with LexA and VirB7 with the activator (Fig. 1). To test whether the reciprocal fusions are also functional, we constructed fusions of VirB7 with LexA and VirB9 with the activator. This combination was not only functional but showed about a fivefold-higher level of β -galactosidase activity (Tables 2 and 3). Because a wide range of activity will allow a more accurate comparison of two fusions, we used this combination for the studies on the identification of the VirB7 interaction domain of VirB9. Several fusions of the acidic activator with defined segments of *virB9* were constructed. The ability of a fusion protein to interact with the LexA-VirB7 fusion was tested by monitoring expression of the *Gall*-LexA_{op}-*lacZ* reporter gene (Table 3). A fusion protein that contains residues 173 to 293 (the end) of VirB9 strongly activated expression of the reporter gene (Table 3), indicating that the first 172 residues of VirB9 are not required for its interaction with VirB7.



FIG. 2. Complementation of *Agrobacterium* A348 Δ B7. Tumor formation on *K. daigremontiana* leaves was monitored as described previously (37). *virB* genes expressed from the plasmid are abbreviated as follows: B7, *virB7*; B8, *virB8*; B9, *virB9*; B7C15S, *virB7C15S*; B7C24S, *virB7C24S*.

Further analysis of the essential region showed that the C-terminal 20 residues are also dispensable (leaving residues 173 to 275). The analysis of additional deletions in the N-terminal region of the interactor domain showed that the deletion of 24 residues in addition to the first 172 (i.e., the first 196 residues) led to a complete loss of interaction. Western blot analysis of the fusion proteins using antihemagglutinin antibodies showed that all strains produced stable fusion proteins (data not shown). Therefore, residues 173 to 275 of VirB9 constitute the minimum interactor domain. Since the disulfide linkage between VirB7 and VirB9 involves VirB9 cysteine 262, a large segment N-terminal to this residue is essential for the interaction with VirB7. The requirement of a relatively large segment of the protein for this interaction suggests that the N-terminal domain lies close to the C-terminal domain in space.

Role of cysteine 24 of VirB7 and cysteine 262 of VirB9 in T-DNA transfer. The ability of the VirB7C24S and VirB9C262S to interact with each other in vivo in yeast suggests that the cysteine residues are not essential for their interaction. If a similar phenomenon occurs in *Agrobacterium*, these two residues could be dispensable for tumor formation. To test this hypothesis, we determined the ability of the *virB7* and *virB9* mutants to complement a deletion mutation in the respective gene in tumor formation assays in potato and *K. daigremontiana* (Table 4; Fig. 2).

Deletion of either *virB7* or *virB9* renders *A. tumefaciens* A348 nontumorigenic (4). In a potato disc assay, the *virB7* deletion mutant *Agrobacterium* A348 Δ B7 could be complemented with a plasmid that constitutively expresses *virB7* and

TABLE 4. Complementation of *Agrobacterium* A348 Δ B7 and A348 Δ B9 with *virB7*, *virB9*, and their mutants

| <i>Agrobacterium</i> strain | <i>virB</i> expression plasmid | Genes expressed from plasmid | Efficiency of tumor formation (%) ^a |
|-----------------------------|--------------------------------|--|--|
| A136 | | | 0 |
| A348 Δ B7 | | | 0 |
| A348 Δ B7 | pAD1424 | <i>virB7</i> , <i>virB8</i> | 47 \pm 3 |
| A348 Δ B7 | pLA21 | <i>virB7</i> , <i>virB8</i> , <i>virB9</i> | 100 |
| A348 Δ B7 | pLA27 | <i>virB7C24S</i> , <i>virB8</i> , <i>virB9</i> | 82 \pm 15 |
| A348 Δ B7 | pLA26 | <i>virB7C15S</i> , <i>virB8</i> , <i>virB9</i> | 3 \pm 3 |
| A348 Δ B9 | | | 0 |
| A348 Δ B9 | pAD1389 | <i>virB9</i> | 136 \pm 48 |
| A348 Δ B9 | pAD1400 | <i>virB9</i> , <i>virB7</i> | 100 |
| A348 Δ B9 | pAD1400C262S | <i>virB9C262S</i> , <i>virB7</i> | 79 \pm 18 |

^a *Agrobacterium* A348 Δ B7 and A348 Δ B9 have deletions in *virB7* and *virB9*, respectively (4). Tumor formation was measured by a potato disc assay (29, 37). The value of the complementing strain with a wild-type gene is set at 100%. The numbers of tumors for the A348 Δ B7 and A348 Δ B9 complementing strains and for A348 were 147 \pm 25, 118 \pm 11, and 503 \pm 118, respectively. The data presented are averages of three independent experiments \pm standard errors.

virB8 from the *virD* promoter (Table 4). When *virB9* was included in the complementing plasmid, a twofold increase in the efficiency of tumor formation was observed. The substitution of *virB7C24S* for *virB7* did not have a significant effect on the complementation ability of the plasmid, indicating that cysteine 24 is not essential for tumor formation. In agreement with a previous study (8), we found that the cysteine in position 15 (*virB7C15S*) is essential for VirB7 function. A high level of constitutive expression is not necessary for complementation because *vir* genes expressed from the inducible *virD* promoter on a low-copy IncP plasmid, pTJS75, are equally efficient in complementation (data not shown). Studies on the complementation of a *virB9* deletion mutant, *Agrobacterium* A348ΔB9, showed that *virB9C262S*, a *virB9* mutant that cannot form a disulfide linkage, can restore the tumor-forming ability of the mutant, indicating that cysteine 262 of VirB9 is not essential for tumor formation.

The disulfide-linked VirB7-VirB9 complex is postulated to play a major role in the assembly of the transport pore (1, 30). VirB9 is unstable in the absence of VirB7 (4, 9). The disulfide linkage between VirB7 and VirB9 has been postulated to be important for the stabilization of VirB9 (9, 30). The stability of a VirB9 homolog, the *B. pertussis* PtlF, is affected by PtlI, the VirB7 homolog. PtlI and PtlF, like VirB7 and VirB9, form a disulfide-linked complex (7). Data presented above demonstrate that VirB7C24S and VirB9C262S, mutants that cannot form the disulfide linkage, are active in tumor formation. The disulfide linkage therefore is not an absolute necessity for protein function. Whether it is essential for the stability of VirB9 cannot be addressed from the results of this study. *Agrobacterium* used in the present study overproduces the plasmid-encoded gene products (1) (data not shown). Consequently, these strains contain a high level of VirB9 and the accumulation of VirB9 in these strains is independent of VirB7.

In potato disc assays, *virB7* and *virB8* complement *Agrobacterium* A348ΔB7 (Table 4). Coexpression of *virB9* increased the efficiency of complementation. The role of *virB9* in the complementation of this particular mutant was apparent when *K. daigremontiana* was used as a host plant (Fig. 2). A previous study showed that on *K. daigremontiana*, tumor formation by *Agrobacterium* A348ΔB7 requires coexpression of *virB7* and *virB8* (4). Under our assay conditions, these results were difficult to reproduce. We observed that *virB7* and *virB8* expressed from the *virD* promoter are unable to complement *Agrobacterium* A348ΔB7; only occasionally, we observed small tumors. A typical result is shown in Fig. 2. To determine the requirements for the complementation of *Agrobacterium* A348ΔB7, we studied the effect of expression of the downstream genes, namely, *virB9*, *virB10*, and *virB11*. We found that in addition to *virB7* and *virB8*, *virB9* is essential for the restoration of tumorigenicity of *Agrobacterium* A348ΔB7 on *K. daigremontiana* (Fig. 2). The *virB7* mutant *virB7C24S*, but not *virB7C15S*, was equally efficient in complementation.

Our observation that *virB9* is essential for the complementation of the *virB7* deletion mutant *Agrobacterium* A348ΔB7 in a tumor formation assay on *K. daigremontiana* leaves does not agree with the previous report that found no such role of *virB9* (4). The two studies differ in the chimeric genes that were used for the expression of the *vir* genes. While the *vir* genes were expressed constitutively from the strong *virD* promoter in our study, the genes were expressed under the control of the *E. coli* lactose operon promoter in the other study. Second, the genes were expressed from a high-copy plasmid in the present study. The difference in the expression level of the proteins, however, cannot account for the discrepancy in the two studies for sev-

eral reasons. First, the native *virB* promoter is a strong promoter, and therefore, under natural induction conditions, the level of the encoded gene products is likely to be high. Second, while the Ti plasmid is present in 1 to 2 copies in the vegetative cell, the copy number of the plasmid is 5- to 10-fold higher in an induced bacterium (36). Third, *vir* genes expressed from the inducible *virD* promoter on the low-copy plasmid pTJS75 are equally efficient in complementation (data not shown). Also, we observed no negative effect of overexpression of the VirB proteins in complementation analysis of several *virB* mutants.

The best interpretation of our results is that a low level of VirB9 in *Agrobacterium* A348ΔB7 is sufficient for tumor formation on potato tubers but not on *K. daigremontiana*. That *Agrobacterium* A348ΔB7 expresses *virB9* at a low level has been reported earlier (9). We suggest that the reduced level of VirB9 in this strain is not only due to the effect of VirB7 on the stability of VirB9 (4) but also is a consequence of the construction of the deletion mutation. If the stability of VirB9 is the only reason, *virB7* expressed from a multicopy plasmid should restore a high level of VirB9 in the cell. Experimental results do not support this prediction (9). *Agrobacterium* A348ΔB7 is also defective in *virB8* since complementation of this mutant requires *virB8* (4; this study).

The aberrant behavior of *Agrobacterium* A348ΔB7 in the present study and that of *Agrobacterium* A348ΔB7 and *Agrobacterium* A348ΔB8 in the previous study (4) led us to reconsider the hypothesis of Ward et al. (38) that the translation of pTiA6 *virB8* initiates at bp 6406 and not at bp 6385. The presence of a ribosome binding site sequence at bp 6378 to 6380 (GGA), the lack of one near bp 6406, and a homologous start site for the pTiC58 *virB8* suggests that bp 6385 is the translation start site of *virB8* (3). If this is true, *Agrobacterium* A348ΔB7, which has a deletion of bp 6332 to 6406, will lose the 7 N-terminal residues of VirB8. This can result in an altered expression of *virB8* and/or may negatively affect the stability of the mutant protein. In Western blot assays, no VirB8 was detected in *Agrobacterium* A348ΔB7 (9; unpublished results). A possible consequence of the modulation of *virB8* expression is a reduction in the expression of the downstream genes.

Studies presented here demonstrate that VirB7-VirB9 interaction does not require a disulfide linkage and, at least under conditions of overproduction, the disulfide bond is dispensable for tumor formation. The nonessential nature of the cysteine residues can account for the lack of conservation of these residues in certain homologs. The VirB7 and VirB9 homologs in the conjugal plasmid pKM101 that encodes homologs of all 11 VirB proteins do not have a conserved cysteine (23). While the disulfide linkage is not essential for tumor formation, it may be involved in the stabilization of the complex and in the maintenance of a rigid orientation of the complex to the bacterial membrane.

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