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Inhibition of VirB-Mediated Transfer of Diverse Substrates from *Agrobacterium tumefaciens* by the IncQ Plasmid RSF1010

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The transfer of DNA from *Agrobacterium tumefaciens* into a plant cell requires the activities of several virulence (*vir*) genes that reside on the tumor-inducing (Ti) plasmid. The putative transferred intermediate is a single-stranded DNA (T strand), covalently attached to the VirD2 protein and coated with the single-stranded DNA-binding protein, VirE2. The movement of this intermediate out of *Agrobacterium* cells and into plant cells requires the expression of the *virB* operon, which encodes 11 proteins that localize to the membrane system. Our earlier studies showed that the IncQ broad-host-range plasmid RSF1010, which can be transferred from *Agrobacterium* cells to plant cells, inhibits the transfer of T-DNA from pTiA6 in a fashion that is reversed by overexpression of *virB9*, *virB10*, and *virB11*. Here, we examined the specificity of this inhibition by following the transfer of other T-DNA molecules. By using extracellular complementation assays, the effects of RSF1010 on movement of either VirE2 or an uncoated T strand from *A. tumefaciens* were also monitored. The RSF1010 derivative plasmid pJW323 drastically inhibited the capacity of strains to serve as VirE2 donors but only partially inhibited T-strand transfer from *virE2* mutants. Further, we show that all the *virB* genes tested are required for the movement of VirE2 and the uncoated T strand as assayed by extracellular complementation. Our results are consistent with a model in which the RSF1010 plasmid, or intermediates from it, compete with the T strand and VirE2 for a common transport site.

Agrobacterium tumefaciens has the capacity to transfer DNA from its Ti plasmid into plant cells, where the DNA is integrated and transcribed (for reviews, see references 5, 22, 58, and 63). In the case of wild-type tumor-inducing (Ti) plasmids, the transferred DNA (T-DNA) encodes two types of proteins: those that synthesize novel amino acid and sugar conjugates (opines) and those that affect the accumulation of plant hormones responsible for the tumorous growth of the transformed cells. The virulence (*vir*) genes of the Ti plasmid are necessary for the production and transfer of the T-DNA. *vir* gene expression is activated by plant wound-released phenolics and sugars, resulting in the accumulation of Vir proteins responsible for T-DNA processing and transfer (58). One of these proteins, VirD2, produces a single-stranded nick at the 25-bp direct repeat border sequences of the Ti plasmid. This is followed by release of a single strand (the T strand) due, apparently, to replacement strand synthesis (45, 49, 50). The T strand is covalently attached to VirD2 and can associate with the single-stranded DNA-binding protein VirE2 (9–11, 15, 39), resulting in the formation of the T (transfer) complex which is thought to be the transferred intermediate (22, 48, 61, 63). In addition to the T-DNA on Ti plasmids, DNA on plasmids that can replicate in *Agrobacterium* cells and carry the border sequences of a Ti plasmid, often termed binary vectors, can be mobilized into plant cells by strains of *Agrobacterium* that carry the *vir* genes (24).

The biochemistry governing movement of the T complex out of the bacterium and into plant cells is not understood. The production of a single-stranded DNA intermediate and the necessity of bacterial attachment to the plant cell for successful DNA transfer (17, 28) suggest that *Agrobacterium*-mediated DNA transfer is similar to conjugative DNA transfer between bacteria (22, 23, 63). This model is further supported by the observations of Buchanan-Wollaston et al. (6, 7), who found that plasmid RSF1010, a broad-host-range plasmid of the IncQ incompatibility group, could be transferred by *A. tumefaciens* to plant cells despite the fact that this plasmid lacks the border sequences upon which the VirD2 protein acts. During bacterial conjugation, the MobA protein of RSF1010 creates a single strand nick at its *oriT* sequence and forms a covalent attachment at this site (19, 38). The *tra* functions of other resident plasmids are then necessary for successful interbacterial DNA transfer (19). Transfer of RSF1010 into the plant cells was shown to be dependent on the *mob* and *oriT* regions and on several of the *vir* genes of the Ti plasmid (6, 7, 53), suggesting that in *Agrobacterium* cells, MobA and *oriT* of RSF1010 are the functional equivalent of VirD2 and the border sequences of the Ti plasmid.

The conjugative model of T-DNA movement proposes that transport of the T complex out of the bacterium and into the plant cell occurs through a multimeric protein channel (63). While no such channel has ever been observed, several pieces of evidence suggest that the proteins encoded by the *virB* operon are likely candidates to compose such a pore. Ten of the eleven genes of this operon are required for virulence (3, 4, 14, 43, 54). As predicted for proteins involved in intercellular transport, all of the VirB proteins so far investigated are localized to the *Agrobacterium* membrane systems (3, 8, 41, 43, 46, 47, 55). While no VirB complex has yet been purified, VirB10 exists in the membrane in a form that is readily cross-linked by chemical reagents into a higher-molecular-weight form (55). The significant sequence homology between the VirB proteins and proteins of IncP, IncW, and IncN conjugative plasmids thought to be involved in formation of conjugal pores (26, 35, 42) supports the hypothesis that the VirB proteins are components of a DNA T complex. Moreover, the fact

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that the *virB* genes are also required for the movement of RSF1010 from *Agrobacterium* cells into plant cells (53) or into other bacteria (2, 20) further implicates them in the process of DNA transfer.

Besides the movement of T complex out of *Agrobacterium* cells, there appear to be at least three other types of macromolecules that can be transferred out of *Agrobacterium* cells and into plant cells: the uncoated T strand (i.e., VirD2-T strand lacking VirE2), VirE2, and VirF. This has been demonstrated by using extracellular complementation assays in which plant cells or explants are exposed to a mixture of two different bacterial strains, one carrying wild-type *vir* genes but no T-DNA and the other carrying mutant *virE2* and wild-type T-DNA. After such coinoculation, the plants develop tumors (34). Several lines of evidence, including the fact that *Agrobacterium* strains lacking a functional Ti plasmid *tra* system still functioned as the VirE donor in these assays, indicated this is not due to bacterial conjugation. On the other hand, wild-type *virE* strains with mutations in either *virB11* or the genes responsible for attachment to plant cells did not function to supply VirE2 (9). Thus, an intimate association of the $virE^+$ bacteria with the plant cell is necessary for extracellular complementation. Movement of VirF from strains carrying pTiC58 was also demonstrated with the use of extracellular complementation assays (29). In the cases of both VirE2 and VirF, transgenic plants overexpressing these proteins formed tumors when inoculated with *virE2* and *virF* mutants, respectively (12, 37). Taken together, these results suggest that VirE2 and VirF can be transported into the plant cell even when not associated with the T strand and that they can function in the plant cell to facilitate transformation. Finally, these same extracellular complementation assays demonstrated that the *virE* mutant strains are capable of moving an uncoated T strand out of *Agrobacterium* cells and into the plant cell.

In earlier studies, we demonstrated that *virB9*, *-10* and *-11* were required for transfer of the IncQ RSF1010 derivative pJW323 from wild-type *A. tumefaciens* A348 (carrying pTiA6) into plant cells. Interestingly, the presence of pJW323 in strain A348 inhibited transfer and/or integration of wild-type T-DNA in the plant cells, as assayed by tumor formation (53). Coordinate overexpression of *virB9*, *-10* and *-11* restored tumorigenicity to A348 containing pJW323, but overexpression of these genes individually or in pairs did not (53). These results suggested that RSF1010 or its transferred intermediate blocks the transfer of the T complex and that in its presence, VirB9, VirB10, and VirB11 proteins are limiting for transfer. In this study, we examined the specificity of RSF1010 inhibition of T-DNA transfer. We monitored the movement of other T-DNAs, in the form of binary vectors, and used extracellular complementation assays to characterize the effect of pJW323 on the movement of VirE2 and uncoated T strands. In addition, we used nonpolar mutations in the *virB* operon to determine whether the various *virB* genes are essential for VirE2 or uncoated T-strand movement. The results suggest that RSF1010 or its transferred intermediate competes with VirE2 and the T strand for limiting sites, most likely the putative VirB complexes, that are necessary for macromolecular transfer.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this work are listed in Table 1.

Reagents and enzymes. The source of acetosyringone (AS) was Aldrich Chemical (Milwaukee, Wis.). Hygromycin B and α-naphthalene acetic acid (NAA) were obtained from Calbiochem (La Jolla, Calif.). Kinetin, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate (BCIP), Tween 20, sodium dodecyl sulfate (SDS), and all other antibiotics except timentin (SmithKline Beecham, Philadelphia, Pa.) were purchased from Sigma Chemical Co. (St. Louis, Mo.). All restriction enzymes, ligase, and Klenow fragment polymerase were obtained from either New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), or Boehringer Mannheim (Indianapolis, Ind.). Arctic shrimp alkaline phosphatase came from United States Biochemical Corp. (Cleveland, Ohio). All enzymes were used as recommended by the manufacturer. DNA fragments were purified from agarose with the Gene Clean II kit from Bio 101 (La Jolla, Calif.). Peroxidase-conjugated goat anti-rabbit immunoglobulins were purchased from Pierce Chemical Co. (Rockford, Ill.). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin was obtained from Bio-Rad Laboratories (Richmond, Calif.). Prestained molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE) were from Gibco BRL (Gaithersburg, Md.). Chemiluminescent immunoblotting reagents were bought from either DuPont, NEN Research Products (Boston, Mass.) or Amersham Corp. (Arlington Heights, Ill.).

Media and growth conditions. *Escherichia coli* and *A. tumefaciens* strains were maintained in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl). Tetracycline was used at a final concentration of 20 μg/ml (plates and broth) for *E. coli* and 5 (plates) and 3 (broth) μg/ml for *A. tumefaciens*. Kanamycin was used at 50 (plates) or 10 (broth) μg/ml, and carbenicillin was used at 100 (plates) or 30 (broth) μg/ml for *Agrobacterium* strains. For analysis of *vir* gene expression, *A. tumefaciens* strains were induced in AB induction medium as described previously (60) with 1% glucose, 1 mM phosphate (pH 5.5), and AS.

Construction of *virE2 virBx* double mutants. Site-specific insertional mutagenesis was used to generate *virE2* mutations in strains already carrying nonpolar mutations in various *virB* genes. Briefly, a 400-bp *Bgl*II-*Sac*I fragment carrying a portion of *virE2* was isolated from pSW108 (59) and cloned into the *Bgl*II and *Sac*I sites of pUC119, yielding pAB113. This plasmid was electroporated into various strains of *A. tumefaciens*, and transformants were selected for carbenicillin resistance. Because pAB113 cannot replicate in agrobacteria, transformants would survive only if the plasmid recombines onto the chromosome or Ti plasmid. Recombination at the *virE2* gene was predicted to yield a truncated form of VirE2 (VirE2′), and this was confirmed in each case by immunoblot analysis (data not shown).

***vir* induction and protein immunoblotting.** Induction of *Agrobacterium* strains for analysis of *vir* expression was carried out as follows. Cells were grown overnight at 30°C in LB with selection. The bacteria were then subcultured into AB induction medium at a starting optical density at 600 nm (OD_{600}) of 0.1 with AS for *vir* induction and grown overnight. The cells were then processed for SDS-PAGE by reading the OD of each sample, pelleting an equivalent OD of cells, and resuspending each sample in 100 μl of SDS sample buffer (12% sucrose, 4% SDS, 0.1 M Tris-HCl [pH 6.8], 5 mM EDTA, 0.04% bromophenol blue, 0.1 M dithiothreitol). The samples were denatured by boiling for 3 min, and 5 μl of each sample was then loaded onto 10% acrylamide gels (18) and subjected to SDS-PAGE. Proteins were transferred from the acrylamide gel to nitrocellulose by electrotransfer by using a Hoefer system. Filters were probed with anti-VirE2 antibodies (15) and developed with either alkaline phosphatase reagent (procedure based on instructions from Bio-Rad) or peroxidase chemiluminescent reagent (procedure based on instructions from Pierce, Amersham, and DuPont, NEN).

Plant transformation assays. Greenhouse-grown *Nicotiana tabacum* cv. Havana 425 plants were used for all leaf explant transformation protocols, using procedures previously described (1). Leaves (fourth youngest visible leaf, ~22 to 25 cm) from vegetative plants (~10 fully expanded leaves) were surface sterilized, cut into 4- by 4-mm pieces, avoiding large veins, and immersed briefly into bacterial solutions. Bacteria were grown overnight in LB plus appropriate antibiotics, pelleted, and resuspended in LB at an OD_{600} of 0.5. In extracellular complementation assays, equal volumes of the two strains (both at an OD_{600} of 0.5) were mixed together, and the leaf pieces were immersed into the mixture. The leaf pieces were then blotted on sterile paper towels and transferred onto plates containing MS medium (30) without hormones and supplemented with 100 μM AS. After 2 days of cocultivation, the leaf pieces were washed in liquid MS containing 200 μg of timentin per ml and plated onto selection plates. Selection plates for tumor formation contained MS medium without hormones supplemented with 200 μg of timentin per ml (to eliminate bacteria). For selection of kanamycin-resistant plant tissue growth, MS medium was supplemented with 10 μM NAA, 1 μM kinetin, 200 μg of timentin per ml, and 100 μg of kanamycin per ml. Selection for hygromycin resistance was done with the same medium as used for kanamycin resistance selection except that hygromycin (10 μg/ml) was used rather than kanamycin. Leaf pieces were scored for tumor or callus growth after 2 to 4 weeks.

RESULTS

RSF1010 inhibits transfer of T-DNA from a binary vector into plant cells. RSF1010 derivative pJW323 contains a neomycin phosphotransferase gene (*nosP-nptII*) that can be delivered into plant cells by wild-type *A. tumefaciens* A348, resulting in kanamycin-resistant plant cell growth (53). Our previous results showed that the presence of pJW323 in A348 blocked this strain's ability to deliver its T-DNA into kalanchoe and

TABLE 1. Plasmids, bacterial strains, and bacteriophage

Plasmid, strain, or phage	Characteristics	Source or reference
Plasmids		
pAB113	Carb^r, *Bgl*II-*Sac*I *virE2* fragment from pSW108 into *Bgl*II-*Sac*I sites of polylinker in pUC119	This study
pBR322	Carb^r cloning vector	Laboratory stock
pED32	Tet^r IncP expression vector carrying *virB* promoter and polylinker	54
pED37	Tet^r, pED32 with *virB9* behind *virB* promoter	14
pED51	Carb^r, 2.8-kb *Bgl*II fragment of *virB* operon (7316–10113[a]) in *Bam*HI site of pBR322	This study
pED52	Tet^r, pED32 with *virB8* behind *virB* promoter	14
pED54	Tet^r, 0.98-kb *Sac*I-*Ssp*I fragment from pJW202 containing *virB6* cloned behind *virB* promoter of pED32	This study
pED56	Kan^r Carb^r, Tn*5virB* in *virB10* of pED51 (~8660[a])	This study
pED59	Kan^r Carb^r, Tn*5virB* in *virB5* of pJW202 (~5050[a])	This study
pED68	Kan^r Carb^r, Tn*5virB* in *virB6* of pJW202 (~5800[a])	This study
pED72	Tet^r, 3.2-kb *Bam*HI fragment containing the *virE* operon from pSW108 cloned at *Bam*HI site behind *lac* promoter of pRK404	This study
pED79	Kan^r Carb^r, Tn*5virB* in *virB4* of pJW202 (~4275[a])	This study
pEND4K	Kan^r Cam^r IncP binary vector, *nos-nptII* plant selectable marker	25
pJW202	Carb^r, 5.15-kb *Bam*HI fragment of *virB* operon (2872–8018[a]) cloned into *Bam*HI site of pUC119	John Ward
pJW323	Kan^r IncQ RSF1010 derivative, *nos-nptII* plant selectable marker	53
pLB1310	Carb^r derivative of pTJS140 with *virB10* behind *virB* promoter	Lois Banta
pMON596	Spec^r IncP binary vector pMON574, with additional restriction sites, *19s-hphA* plant selectable marker	27
pRK404	Tet^r IncP cloning vector carrying *lac* promoter and linker	16
pSW108	Carb^r, 3.2-kb *Xho*I fragment carrying *virE* operon in *Sal*I site of pUC7	59
pUC119	Carb^r cloning vector	Laboratory stock
A. tumefaciens strains		
A136	C58 cured of its Ti plasmid	56
A348	A136 containing pTiA6NC	21
Ax56	Kan^r, *virB10*::Tn*5virB* replacing *virB10* in pTiA6 of A348	This study
Ax42	Kan^r, *virB9*::Tn*5virB* replacing *virB9* in pTiA6 of A348	14
Ax46	Kan^r, *virB8*::Tn*5virB* replacing *virB8* in pTiA6 of A348	14
Ax68	Kan^r, *virB6*::Tn*5virB* replacing *virB6* in pTiA6 of A348	This study
Ax59	Kan^r, *virB5*::Tn*5virB* replacing *virB5* in pTiA6 of A348	This study
Ax79	Kan^r, *virB4*::Tn*5virB* replacing *virB4* in pTiA6 of A348	This study
A348::*virE2*	Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of A348	This study
Ax56::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax56	This study
Ax42::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax42	This study
Ax46::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax46	This study
Ax68::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax68	This study
Ax59::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax59	This study
Ax79::*virE2*	Kan^{r}Carb^r, pAB113 cointegrated into *virE2* in pTiA6 of Ax79	This study
LBA4404	Sm^r, Ach5 chromosome, pTiAch5 carrying deletion of T-DNA	33
358mx	Carb^r, A348 with Tn*3*HoHo1 in *virE*	44
E. coli strains		
DH5α	F$^-$ *recA endA1 gyrA96 thi-1 hsdR17* (r_{K^-} m_{K^+}) *supE44 relA1*	Clontech
HB101	F$^-$ *hsdS20* ($r_B{}^-$ $m_B{}^-$) *supE44 recA13 ara-14* λ$^-$ *galK2 lacY1 proA2 rpsl120 xyl-5 mtl-1* $r_B{}^-$ $m_B{}^-$	Clontech
MC1061	*F$^-$ araD139* Δ(*ara-leu*)*7696* Δ*lacY74 galU galK hsr hsm$^+$*	Clontech
MC1061::Tn*5virB*	MC1061 carrying Tn*5virB*	This study
Bacteriophage λ::Tn*5virB*	λ containing Tn*5virB*	14

[a] Numbers in parentheses indicate nucleotide positions in the *virB* operon as reported by Ward et al. (51, 52).

tobacco (53). To determine whether this inhibition was due to some specific characteristic of pTiA6 of strain A348 or to a more general disruption of the transfer process, we tested the effect of pJW323 on movement of T-DNA from a binary vector, pMON596, into plant cells. pMON596 was chosen because (i) it contains the border sequences from a nopaline- rather than an octopine-type Ti plasmid and (ii) its T-DNA contains a hygromycin phosphotransferase A (*hphA*) gene under the control of the 19S promoter of the cauliflower mosaic virus (27), thereby allowing us to monitor its movement as well as that of pJW323. LBA4404, a "disarmed" strain of *Agrobacterium* that contains the *vir* genes but not the T-DNA, delivered either vector, separately, into plant cells, as indicated by the ability of leaf explants exposed to either LBA4404(pJW323) or LBA4404(pMON596) to form callus in the presence of kanamycin and hygromycin, respectively (Table 2). In contrast, when LBA4404 contained both plasmids, the cocultivated leaf explants produced kanamycin-resistant callus but little or no hygromycin-resistant callus (Table 2). This result demonstrated that the presence of pJW323 inhibits the transfer of

TABLE 2. Effect of pJW323 on transfer of a standard binary vector T-DNA from strain LBA4404

Bacterial strain	Transformation[a] (% of explants forming actively growing callus [n = 20–25])	
	Kanamycin resistance	Hygromycin resistance
LBA4404	0	0
LBA4404(pMON596)	0	100
LBA4404(pJW323)	29	0
LBA4404(pJW323, pMON596)	34	4

[a] Leaf explants were cocultivated for 2 days with the various strains as described in Materials and Methods and then transferred to MS medium containing 10 μM NAA, 1 μM kinetin, 500 μg of cefotaxime per ml, and 200 μg of vancomycin per ml plus either 100 μg of kanamycin per ml or 10 μg of hygromycin per ml.

T-DNA from a binary vector carrying the right border of the nopaline plasmid, pTiC58, as well as the wild-type T-DNA from pTiA6.

Effect of pJW323 on extracellular complementation of the *virE* mutant strain 358mx. In extracellular complementation assays, plant tissues are exposed to a mixture of a strain producing VirE2 but lacking T-DNA and a *virE* mutant strain containing T-DNA. The plant tissues are subsequently monitored for T-DNA activity, as assayed by tumor formation (9, 34). We tested strains of LBA4404 carrying either pJW323 or a standard binary vector, pEND4K, for the ability to function in extracellular complementation assays when mixed with the *virE* mutant strain 358mx (44) as the T-DNA-donating strain (Fig. 1; Table 3). Leaf explants cocultivated with a 1:1 mixture of LBA4404 and 358mx readily formed tumors (Fig. 1A), as expected when the T-DNA from 358mx is integrated into the plant genome, while neither strain alone induced such growths (Fig. 1B and C). LBA4404 carrying a standard binary vector, pEND4K, also functioned as a source of VirE2 (Fig. 1D). In contrast, the ability of LBA4404 to function in the extracellular complementation test was significantly inhibited when it also carried pJW323 (Fig. 1E; Table 3). These results suggested that pJW323 interferes with the ability of VirE2 either to exit the bacterium or to function in the plant cell. Both LBA4404 (pEND4K) and LBA4404(pJW323) were, however, capable of delivering their *nptII* genes into plant cells, as ascertained by kanamycin resistance, and this occurred when these strains were cocultivated with leaf tissues in the presence or absence of 358mx (Table 3).

TABLE 3. Effect of pJW323 on capacity of LBA4404 for extracellular complementation of *virE* mutant strain 358mx

VirE2 donor strain	Transformation[a] (% of explants forming actively growing callus [n])			
	−358mx		+358mx	
	Tumors	Kanamycin resistance	Tumors	Kanamycin resistance
None			0 (24)	0 (8)
LBA4404	0 (24)	0 (16)	100 (23)	0 (15)
LBA4404(pEND4K)	0 (23)	100 (15)	96 (24)	93 (16)
LBA4404(pJW323)	0 (21)	63 (24)	8 (24)	71 (21)

[a] Leaf explants were cocultivated for 2 days with the complementing strain in the presence or absence of *virE* strain 358mx (1:1 ratio) and then transferred to MS medium containing 200 μg of timentin per ml and 200 μg of vancomycin per ml for tumor assay or MS medium containing 200 μg of timentin per ml and 200 μg of vancomycin per ml plus 10 μM NAA, 1 μM kinetin, and 100 μg of kanamycin per ml for the kanamycin resistance assay. Explants were scored for callus growth on these media after 21 days.

To characterize its effect on uncoated T-strand transfer, pJW323 was electroporated into the *virE* mutant strain 358mx, and the resultant strain, 358(pJW323), was used in extracellular complementation assays. pJW323 from this strain transformed plant cells, as indicated by kanamycin-resistant growth, only when LBA4404 was also present in the cocultivation (Fig. 2), suggesting VirE2 is necessary for this event (also see

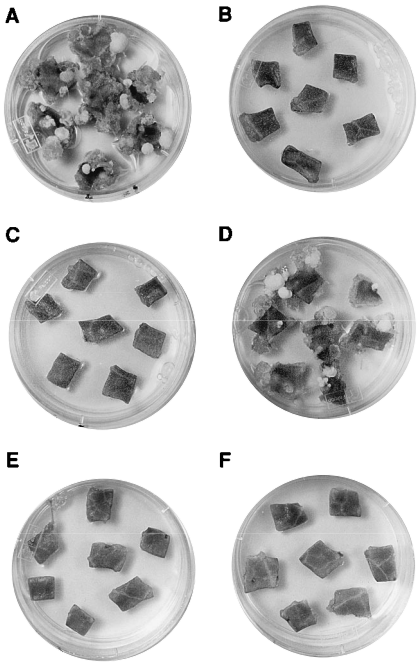

FIG. 1. Extracellular complementation of *virE* mutant strain 358mx by strain LBA4404 carrying pEND4K or pJW323. Leaf explants cocultivated with various strains of *A. tumefaciens* for 2 days and then incubated for 3 weeks on hormone-free MS medium containing 200 μg of timentin per ml: (A) 358mx plus LBA4404; (B) LBA4404; (C) 358mx; (D) 358mx plus LBA4404(pEND4K); (E) 358mx plus LBA4404(pJW323); (F) LBA4404(pJW323).

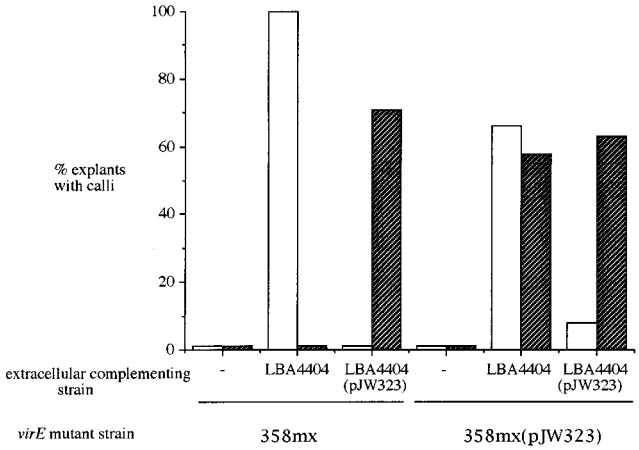

FIG. 2. Effect of pJW323 on movement of uncoated T strand from *virE* mutant strain 358mx. Leaf explants were cocultivated with various strains of *A. tumefaciens* for 2 days and then incubated on either hormone-free MS (open bars) or MS containing 10 μM NAA, 1 μM kinentin, and 100 μg of kanamycin per ml (cross-hatched bars). The percentage of explants forming calli was determined after 3 weeks (n = 20 to 24).

TABLE 4. Effect of overexpression of VirE2 or extracellular complementation on transformation by strains carrying pJW323

T-DNA donor strain[a]	Tumors[b]			Kanamycin resistance[c]		
	−LBA4404	+LBA4404	R[d]	−LBA4404	+LBA4404	R
358mx	0 ± 0	7.2 ± 0.7		0 ± 0	0 ± 0	
358(pJW323)	0 ± 0	3.8 ± 0.5		0 ± 0	1.1 ± 0.5	
358(pED72)	5.3 ± 0.5	6.5 ± 0.6	1.2	0 ± 0	0 ± 0	
358(pJW323, pED72)	0.1 ± 0.1	0.5 ± 0.1	5.0	0.2 ± 0.1	1.7 ± 0.1	8.5
A348	6.3 ± 0.5	10.0 ± 0.8	1.6	0 ± 0	0 ± 0	
A348(pJW323)	0.7 ± 0.2	1.4 ± 0.3	2.0	0.1 ± 0.1	3.9 ± 0.6	39
A348(pJW323, pED72)	0.4 ± 0.1	0.5 ± 0.1	1.3	0.1 ± 0.1	1.3 ± 0.3	13

[a] Leaf explants were cocultivated either with T-DNA donor strains or with a 1:1 mixture of T-DNA donor strains and LBA4404 as described in Materials and Methods.

[b] Leaf explants were transferred to selection medium containing hormone-free MS medium supplemented with 200 μg of timentin per ml. The mean number ± standard error of individual calli of >1 mm in diameter on leaf explants described in footnote *c* was determined after 14 days. $n = 20$ to 24.

[c] Leaf explants were transferred to selection medium containing MS medium supplemented with 10 μM NAA, 1 μM kinetin, 200 μg of timentin per ml, and 100 μg of kanamycin per ml. The mean number ± standard error of individual calli of >1 mm in diameter on leaf explants was determined after 14 days. $n = 20$ to 24.

[d] R, ratio of +LBA4404 to −LBA4404.

below). Surprisingly, these extracellular complementation assays revealed that transfer of the T-DNA from 358mx into plant cells (tumor formation) was not inhibited to the same extent in 358(pJW323) as in wild-type A348 containing pJW323 (53) (Table 4). Tumor formation was, however, significantly reduced when the extracellular complementation of 358(pJW323) was attempted with LBA4404(pJW323) as the source of wild-type VirE2 (Fig. 2).

Overexpression of VirE2 does not overcome inhibition of T-DNA transfer by pJW323. The results described above indicated that the presence of pJW323 in agrobacteria inhibited T-DNA transfer when VirE2 was present but inhibited it to a lesser extent when this protein was absent from the bacterium but instead provided extracellularly. In addition, pJW323 blocked VirE2 transfer as monitored by extracellular complementation assays. These results can be interpreted to mean that VirE2 becomes limiting in the presence of pJW323. We therefore sought to determine whether T-DNA transfer from wild-type strains carrying pJW323 could be restored by overexpressing VirE2. The entire *virE* operon, including its own promoter, was ligated into a *Bam*HI site just downstream of the *lac* promoter on the multicopy, broad-host-range plasmid pRK404 (16), yielding pED72. Expression from this construct in agrobacteria is constitutive from the *lac* promoter, because no *lac* repressor is present, and AS is inducible from the *virE* promoter. pED72 was electroporated into various strains, which were then tested for VirE2 production and virulence characteristics. Immunoblot analysis indicated that AS induction of strain 358mx(pED72) resulted in high levels of VirE2 production (Fig. 3A). The presence of VirE2 in the uninduced lanes of strains carrying pED72 is due to constitutive expression from the *lac* promoter of the vector. The high-molecular-weight cross-reactive band is a translational fusion between Ti plasmid-encoded *virE2* and Tn*3*Hoho1 sequences (9). VirE2 was present in uninduced A348(pJW323, pED72) cells and more abundant in induced cells of this strain than in A348 or A348(pJW323) cells (Fig. 3B), although precise quantitation was not possible in this type of analysis. Virulence assays show that 358(pED72) caused tumors on tobacco (Table 4), confirming that pED72 produced functional VirE2. In contrast, the presence of pED72 in A348(pJW323) did not restore virulence (Table 4). Interestingly, when pJW323 and pED72 were present in the *virE* mutant 358mx, the strain was severely attenuated but capable of delivering pJW323, as indicated by the acquisition of kanamycin resistance by the plant tissues. Thus, the presence of pED72 and pJW323 in 358mx caused it to exhibit the same phenotype as wild-type strain A348 carrying pJW323. These data also demonstrated that VirE2 is, in fact, required for transformation of plant cells by pJW323.

Effect of extracellular complementation on strains carrying wild-type VirE2 and pJW323. We next tested the possibility that extracellular complementation could restore the capacity of T-DNA transfer to *virE*$^+$ strains carrying pJW323. Various *virE*$^+$ strains containing both T-DNA and pJW323 were mixed

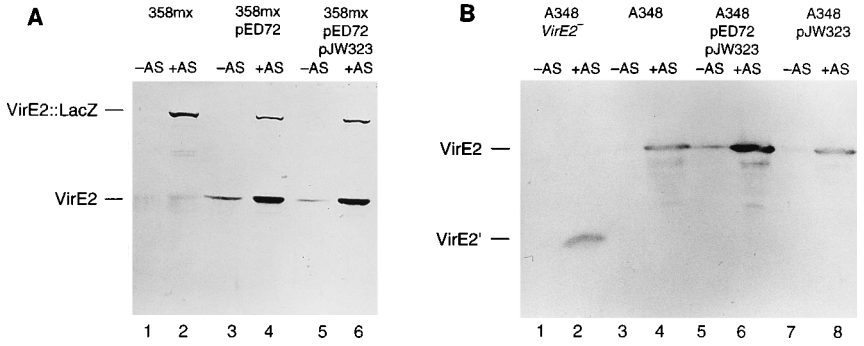

FIG. 3. Immunoblot analysis of VirE2 accumulation in various strains of *A. tumefaciens*. (A) Cultures of *virE* mutant strain 358mx carrying the indicated plasmids were grown as described in Materials and Methods in the presence or absence of 100 μM AS for 18 h, and protein extracts were prepared, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-VirE2 antibodies (15). (B) Cultures of *virE* mutant strain A348::*virE2* and wild-type strain A348 carrying various plasmids were grown and analyzed as for 358mx except that 5 μM AS was used in the induction medium.

TABLE 5. *virB* genes are required for extracellular complementation

VirE2 donor strain[a]	Kanamycin resistance (% of explants forming actively growing calli after 21 days [*n*])[b]	
	−358(pEND4K)	+358(pEND4K)
None		0 (12)
A348	0 (22)	92 (24)
A348::*virE2*	0 (20)	0 (22)
Ax56	0 (11)	0 (14)
Ax56(pLB1310)	0 (14)	67 (12)
Ax42	0 (15)	0 (16)
Ax42(pED37)	0 (13)	91 (11)
Ax46	0 (13)	0 (12)
Ax46(pED52)	0 (12)	77 (13)
Ax68	0 (14)	0 (11)
Ax68(pED54)	0 (13)	100 (12)
Ax59	0 (14)	0 (14)
Ax79	0 (13)	0 (15)

[a] Leaf explants were cocultivated with either the complementing strain or a 1:1 mixture of the complementing strain plus 358(pEND4K) for 2 days as described in Materials and Methods.

[b] After cocultivation, leaf explants were transferred to MS medium supplemented with 10 μM NAA, 1 μM kinetin, 200 μg of timentin per ml, and 100 μg of kanamycin per ml.

with LBA4404, cocultivated with tobacco leaf explants, and subsequently analyzed for tumor formation and kanamycin resistance. The results demonstrated that the extracellular complementation of A348(pJW323) with LBA4404 caused a very small but detectable increase in tumor formation (Table 4). In seven different experiments, the effect ranged from no stimulation to the slight increase seen here. This same result was observed when 358(pJW323, pED72) was used as the T-DNA donor (Table 4). Similar results were obtained when LBA4404 (pJW323, pMON596) was cocultivated with LBA4404: only a small increase in hygromycin-resistant calli was observed (data not shown). In all three cases, plant transformation frequency, and any observed increase in frequency, achieved by extracellular complementation was significantly less than that observed in such assays monitoring movement of T-DNA from *virE* mutant strains [e.g., 358(pJW323)]. In contrast to the results of the T-DNA transfer assays, extracellular complementation dramatically increased transfer of pJW323 from the VirE2-containing strains A348(pJW323) and 358(pJW323, pED72) (Table 4). This increase in pJW323 transfer to plant cells by A348(pJW323) also was observed when it was mixed with A348 but not when *virE2* mutant strain 358mx or A348::*virE2* was used as the complementing strain (data not shown).

***virB* genes necessary for extracellular complementation.** The experiments presented above suggest that pJW323 or its transferred intermediate blocked either T-complex or VirE2 export but had a less severe effect on uncoated T-strand transfer from *virE* mutants. One mechanism that could account for this specificity is that different *virB* genes are required for transfer of different substrates. We determined whether the various *virB* genes are required specifically for VirE2 transport by comparing the effects of nonpolar *virB* mutations on virulence with the ability of the strain to function in extracellular complementation of a *virE2* mutant. For this study, we used strains Ax56, Ax42, Ax46, Ax68, Ax56, and Ax79 carrying Tn*5virB* insertions in *virB10*, *-9*, *-8*, *-6*, *-5*, and *-4*, respectively. This transposon carries a *virB* promoter at its terminal repeat and, when inserted in the proper orientation in the *virB* operon, will result in AS-inducible transcription of the downstream open reading frames (14). Each of these strains responded to phenolics by expressing all downstream proteins examined (VirB8, VirB9, VirB10, and VirB11, as ascertained by immunoblotting), and each is avirulent in tobacco leaf disc assays (reference 14 and data not shown). Because both 358mx and the Ax strains carry wild type T-DNA, delivery of the binary vector pEND4K from 358mx was monitored so that T-DNA originating from the *virE* mutant strain could be identified. None of the *virB* mutants functioned as the VirE2 donor in extracellular complementation assays. Production of kanamycin-resistant plant callus tissue by infected leaf pieces occurred only when the Ax strains used for complementation of 358(pEND4K) also carried the expression plasmid with the corresponding *virB* gene (Table 5). Thus, *virB4*, *-5*, *-6*, *-8*, *-9*, and *-10* are required for extracellular complementation, as is *virB11* (8). These experiments also demonstrated that the presence of full-length T-DNA in a VirE2-containing strain does not prevent that strain from serving as VirE2 donor: strain A348 served to complement 358(pEND4K).

In a second series of experiments, we determined whether specific *virB* genes are required for movement of the uncoated T strand out of *Agrobacterium* cells. This was accomplished by creating an insertion mutation in the *virE2* gene of each of the nonpolar Tn*5virB* mutants described above as well as in the wild-type strain A348 (see Materials and Methods). The absence of full-size VirE2 was confirmed in each case by immunoblot analysis (data not shown), although all such strains carried the truncated VirE2′ fragment predicted by the recombination strategy. An example of this is shown for A348::*virE2* (Fig. 3B). These strains were then used as T-strand donor in extracellular complementation assays using LBA4404 as the source of VirE2. In each case, tumors were not formed when the *virE2* mutant strain also carried a nonpolar mutation in a *virB* gene (Table 6). Thus, all of the *virB* genes tested were required for T-strand movement.

DISCUSSION

The experiments presented in this report characterized the effect of pJW323, a derivative of the IncQ plasmid RSF1010, on macromolecular movement from *A. tumefaciens* and the role of the *virE2* and *virB* genes in such movement. Specifically, we found that (i) pJW323 interfered with transformation of plant cells by T-DNA of a binary vector (Table 2); (ii) the presence of pJW323 inhibited the ability of strains carrying wild-type VirE2 to function in extracellular complementation of *virE2* mutant strains (Fig. 1; Table 3); (iii) T-DNA transfer

TABLE 6. *virB* genes are required for uncoated T-strand transfer

T-strand donor strain[a]	Tumor formation (% of explants forming actively growing calli after 15 days [*n*][b])	
	−LBA4404	+LBA4404
None		0 (17)
A348::*virE2*	0 (22)	96 (23)
Ax56::*virE2*	0 (23)	0 (22)
Ax42::*virE2*	0 (24)	0 (24)
Ax46::*virE2*	0 (24)	0 (23)
Ax68::*virE2*	0 (24)	0 (24)
Ax59::*virE2*	0 (24)	0 (24)
Ax79::*virE2*	0 (23)	0 (24)

[a] Leaf explants were cocultivated with either the T-donor strain or a 1:1 mixture of the donor strain plus LBA4404 for 2 days as described in Materials and Methods.

[b] After cocultivation, leaf explants were transferred to MS medium supplemented with 200 μg of timentin per ml.

from *virE2* mutant strains (as assayed by extracellular complementation) was significantly less inhibited by pJW323 than was T-DNA transfer from wild-type strains carrying this plasmid (Fig. 2; Table 4); (iv) in contrast to an earlier report on the transfer of RSF1010 derivatives from *Agrobacterium* cells to plant cells (7), plant transformation by pJW323 was dependent on VirE2 (Table 4); (v) overexpression of VirE2 in strains carrying pJW323 did not restore their capacity to transfer T-DNA and had little effect on pJW323 transfer (Table 4); (vi) transformation by pJW323, but not the T-DNA, from wild-type *A. tumefaciens* to plant cells was dramatically increased by extracellular complementation using *virE2*$^+$ but not *virE2* mutant strains (Table 4); and (vii) all *virB* genes tested are necessary for the capacity of the strain to serve as VirE2 donor or T-strand donor in extracellular complementation assays (Tables 5 and 6).

One mechanism that could account for the capacity of pJW323 to inhibit both wild type T-DNA transfer and extracellular complementation of *virE2* mutants is that the putative single-stranded pJW323 intermediate (R strand) is more abundant than the T strand, sequestering VirE2 and thereby limiting its availability for other activities. Using Southern blot analysis, we estimate that pJW323 is present in agrobacteria at ~20 copies per cell, whereas the IncP binary vector pEND4K is present at 5 to 10 copies per cell (data not shown). The Ti plasmid is present at one to two copies per cell (40, 62). However, it seems unlikely that the observed effects of pJW323 can be ascribed simply to an abundant putative transferred intermediate binding all of the available VirE2. Overexpression of VirE2 in agrobacteria did not increase transformation by either pJW323 or the T-DNA (Table 4), as would be expected if there was competition between T and R strands inside the bacteria for limiting VirE2. In contrast, the observation that pJW323 transfer, but not T-DNA transfer, from wild-type strains was substantially increased by extracellular complementation with VirE2-producing strains indicates that pJW323 can preferentially take advantage of extracellular VirE2. A working model that is consistent with these results is presented in Fig. 4. This model suggests that the transferred DNA intermediate is actually uncoated T strand (or R strand in the case of pJW323) and that VirE2 is transferred to plant cells independent of these intermediates. Additionally, the model predicts that the pJW323 intermediate sequesters some limiting factor related to transfer, perhaps part of the *virB* complex.

The first feature of the model is based on four sets of results. First, the T-DNA and pJW323 can move out of *virE2* mutant strains in extracellular complementation assays. Second, *virE2* mutant strains are virulent when inoculated onto plants overexpressing VirE2 (12). Both of these results strongly support the hypothesis that uncoated T strand can leave the bacterium. Third, Yusibov et al. (61) demonstrated the presence of the T strand in plant cells infected by *virE* mutant strains and suggested that VirE2 functions inside the plant cell to protect this bacterial DNA from degradation. These results clearly demonstrate that VirE2 in the bacterium is not necessary for movement of the T strand to the plant cell. Finally, intracellular overexpression of VirE2 does not increase transfer of pJW323, whereas extracellular VirE2 does. This result indicates that intracellular VirE2 is not a limiting factor in pJW323 transfer, consistent with the hypothesis that this plasmid moves out of *Agrobacterium* cells separately from VirE2.

While movement of VirE2 out of the bacteria and into the plant has not been demonstrated directly in extracellular complementation experiments, this remains the most likely explanation for the success of such assays. The possibility that either a *virE* operon or VirE2 itself moves into the T-DNA donor is unlikely: overexpression of VirE2 in A348(pJW323) did not increase plant transformation by pJW323, whereas extracellular complementation of A348(pJW323) by *virE2*$^+$ (but not *virE2* mutant) strains did (Table 4). This experiment conclusively demonstrates that VirE2 moving into the pJW323-donating bacterium cannot be responsible for extracellular complementation. Our data also demonstrated that the presence of T-DNA did not interfere with the capacity of a strain to serve as VirE2 donor in extracellular complementation, confirming earlier observations by Christie et al. (9). Given the affinity of VirE2 for single-stranded DNA (10, 39), it seems unlikely that VirE2 displaced from the T complex in the plant cell could serve as the sole source of extracellular VirE2. VirE2 inside agrobacteria following *vir* induction by phenolics is an abundant protein sufficient, minimally, to coat the entire T strand (63). Our results suggest that more VirE2 is made than would be needed to bind to the T strand within the bacterium, and this is exported into the plant cell. The concept that the VirB complex serves to translocate proteins is also supported by the observation that the VirB proteins have a high degree of homology to the Ptl proteins of *Bordetella pertussis* that are responsible for toxin secretion from this gram-negative bacterium (42, 57). In addition, protein movement from conjugal donor to recipient in bacteria has also been demonstrated (36).

Competition between the various substrates within *Agrobacterium* cells for some limiting factor related to macromolecular

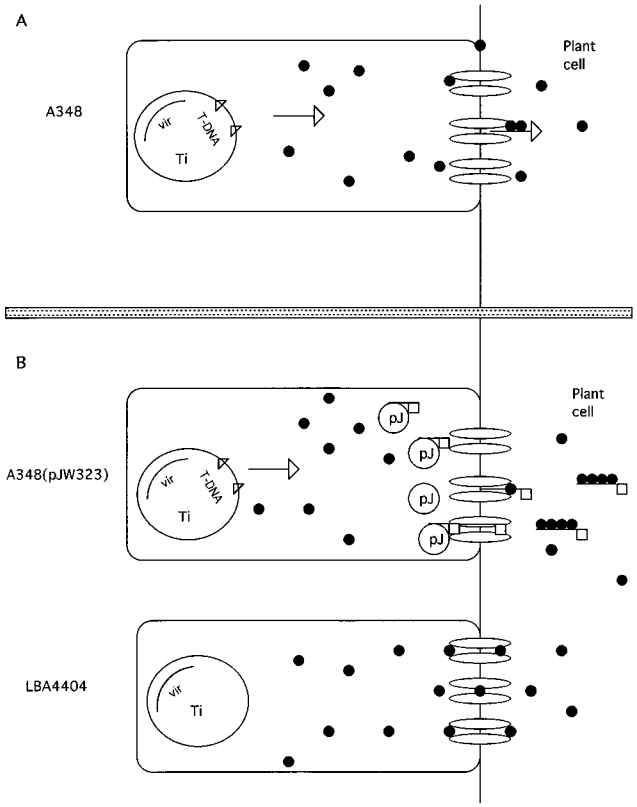

FIG. 4. Model of *A. tumefaciens*-mediated transformation (A) and pJW323-mediated inhibition of T-strand and VirE2 transfer into plant cells, with pJW323 transfer restored by extracellular complementation by LBA4404. See text. Ti, Ti plasmid; pJ, pJW323; ▷, T-DNA border sequences; ●, VirE2; ——▷, T strand; ——□, R strand; ⋈, VirB complex.

transport is suggested by the experimental evidence. For example, extracellular VirE2 dramatically stimulated pJW323 movement from *Agrobacterium* cells into plant cells but had very little effect on T-strand movement from these strains (Table 4). Assuming that both the T strand and the pJW323 intermediates are present in the bacterium, this result indicates that the pJW323 intermediate has better access to the extracellular VirE2, a situation that could easily develop if the pJW323 intermediate sequesters a limiting export function. The inability of *virE2*$^+$ strains carrying pJW323 to serve as a source of VirE2 in extracellular complementation assays indicates that this plasmid also competes with VirE2 for export. Finally, the fact that pJW323 was much less effective at inhibiting T-strand transfer from *virE* mutant strains than from wild-type strains (Fig. 2; Table 4) suggests that it is not disrupting production of functional T-DNA intermediates. Rather, the lack of VirE2 in such strains may reduce competition for limiting transport sites, allowing both T-strand and R-strand intermediates access to extracellular VirE2, thereby facilitating transformation.

The putative VirB transport complex represents the most likely site of competition between the different transported macromolecules. Genetic data presented here demonstrate that VirB proteins are required for the VirE2 donor and the T-strand donor to function in extracellular complementation assays. pJW323 transfer to plant cells also requires the *virB* genes (53). The proposal that pJW323 outcompetes the T-DNA for *virB* products is most clearly supported by the observation that coordinated overexpression of *virB9*, *-10*, and *-11* relieved pJW323-mediated inhibition of tumor formation (53). The relatively high copy number (~20) of pJW323 could be an important component of this competition, although IncP plasmids, with ~5 to 10 copies per cell, have no effect on extracellular complementation or T-DNA transformation by wild-type strains. Perhaps more importantly, pJW323 is probably present in *Agrobacterium* species, even in the absence of *vir* induction, as it is in *E. coli*, in which the relaxed, MobA-capped intermediate of RSF1010 is found constitutively (31). As potentially limiting VirB proteins are made after *vir* induction, they may be sequestered by this type of intermediate.

The transfer of pJW323 out of *Agrobacterium* cells into plant cells is less efficient than is transfer of wild-type T-DNA. For example, LBA4404(pEND4K) and LBA4404(pMON596) routinely exhibited higher plant transformation frequencies than LBA4404(pJW323) or A348(pJW323) (Tables 2 to 4). This observation, coupled with the fact that plant transformation by pJW323, but not by T-DNA, can be dramatically increased by extracellular complementation, suggests that the pJW323 intermediate may inefficiently depart from the transport apparatus unless VirE2 is present outside the bacterium. Alternatively, the T strand, R strand, and VirE2 may all enter the plant cell, but the more abundant R strand may sequester the available VirE2, even the excess provided by extracellular complementation. If this is the case, extracellular complementation of the VirE2-producing A348(pJW323) should result in a frequency of T-DNA transformation higher than that observed in extracellular complementation of the *virE* mutant 358 (pJW323), which must rely solely on the extracellular VirE2. The results are just the opposite: 358mx(pJW323) demonstrated a significantly greater T-DNA transformation frequency than did A348(pJW323) in extracellular complementation assays (Fig. 2; Table 4). Thus, competition between a low-abundance T strand and higher-abundance R strands in the plant cell for VirE2 is not likely to be the basis of low frequency of T-DNA transformation by strains such as A348(pJW323).

Our data indicate that the VirB proteins are necessary for *virE2*$^+$ strains to function in extracellular complementation of *virE2* mutants. This finding provides genetic evidence for interaction between the putative VirB apparatus and VirE2. Because transport of uncoated T strand from *virE* mutants was less affected by pJW323 than that of VirE2 or T-DNA from wild-type strains carrying this plasmid, we tested the hypothesis that individual *virB* genes may be specifically involved in transport of one substrate or another. *virB4*, *-5*, *-6*, *-8*, *-9*, *-10*, and *-11* were all required for successful extracellular complementation (Table 5 and reference 9), thereby implicating them in the export of VirE2 into plant cells. Given the findings that *virE2* is required for virulence and that the extracellular complementation assays indicated that *virB* genes are necessary for VirE2 movement, it became apparent that no test addressing the involvement of the *virB* genes in movement of the uncoated T strand had been reported. To determine whether any *virB* genes are specifically required for uncoated T-strand transfer, we created the *virB virE* double mutants for use as T-DNA donors in extracellular complementation assays. These experiments showed that *virB4*, *-5*, *-6*, *-8*, *-9*, and *-10* were all required for transfer of uncoated T strands into plant cells (Table 6). Thus, the variable effect of pJW323 on movement of T-DNA from wild-type or *virE* mutant strains is not likely to be the result of interaction of this plasmid with a VirB protein(s) that is specifically involved in transport of one particular substrate.

In summary, the experiments presented in this report are consistent with a model in which various substrates (R strand, T strand, and VirE2) utilize a common transport site and that the transferred intermediate of pJW323 has an advantage over either VirE2 and VirD2-T strand in this accessing this site. The most obvious candidate site of competition is the hypothetical transfer complex encoded by *virB* and, potentially, *virD4* (32). Besides being dependent on the *virB* genes for transfer to plant cells, RSF1010 derivatives have also been shown to be mobilized from one *Agrobacterium* strain to another in a *virB*-dependent manner (2) but are not conjugally transferred between bacteria by the Ti *tra* system (13). These results indicate that RSF1010 preferentially interacts with the *virB*-encoded transfer system. Our results lead us to suggest that the transferred intermediate of pJW323 somehow sequesters the putative transport apparatus and does so in a fashion that leaves this intermediate in a state that is more likely to take advantage of extracellular VirE2 than the T-DNA intermediate made in the same bacterium.

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