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# Activity of the *Agrobacterium* T-DNA transfer machinery is affected by *virB* gene products

(crown gall/IncQ plasmid/conjugal transfer/oncogenic suppression)

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ABSTRACT **The *oriT* (origin of transfer) sequence and *mob* (mobilization) genes of plasmid RSF1010 can functionally replace transfer DNA (T-DNA) borders to generate an RSF1010 intermediate transferable to plants through activities of the tumor-inducing (Ti)-plasmid virulence (*vir*) genes of *Agrobacterium tumefaciens*. Because the Ti plasmid *virB* gene products are hypothesized to form a membrane-localized T-DNA transport apparatus, we investigated whether specific *virB* genes were needed for RSF1010 transfer. Here we report that transformation of *Nicotiana tabacum* leaf explants by an RSF1010-derivative plasmid (pJW323) requires the essential virulence genes *virB9*, *virB10*, and *virB11*, consistent with the hypothesis that both the T-DNA and RSF1010 transfer intermediates utilize the same transport machinery. Further, while pJW323 is transferred into plant cells by *Agrobacterium* strains harboring both pJW323 and pTiA6, the initiation of crown gall tumors (i.e., T-DNA transfer) is greatly suppressed. Coordinate overexpression of the *virB9*, *virB10*, and *virB11* gene products relieves pJW323-mediated oncogenic suppression and restores tumorigenicity, but does not increase the transfer frequency of pJW323 into plant cells. We propose that the *virB9*, *virB10*, and *virB11* gene products function coordinately and stoichiometrically to enhance DNA transfer in a fashion specific for the T-DNA intermediate.**

During the initiation of crown gall tumorigenesis in plants by *Agrobacterium tumefaciens*, a discrete DNA molecule [transfer DNA (T-DNA)] is mobilized out of the large bacterial tumor-inducing (Ti) plasmid, transferred across the bacterial and plant cell walls into the plant cell nucleus, and stably integrated into the plant genome (reviewed in refs. 1–3). The early stages of this process require two distinct genetic regions on the Ti plasmid: the T-DNA borders and the virulence (*vir*) genes. The borders are 25-base-pair (bp) imperfect direct repeats that delimit the ends of the T-DNA. Proteins encoded by the *vir* genes function to (*i*) recognize that an appropriate host site is available, (*ii*) generate the T-DNA intermediate that is transferred, and (*iii*) transport this intermediate across the bacterial envelope.

The T-DNA transfer process is analogous to the process of bacterial plasmid conjugation in several respects (3, 4). During both processes single-stranded DNA (ssDNA) is thought to be the transfer intermediate. In the case of *Agrobacterium*, this DNA (the T-strand) is coated with the ssDNA-binding protein (SSB) VirE2 (5–8) and is capped at its 5′ end by the VirD2 protein (9–11) to form the "T-complex" (8). Evidence of functional similarity between these DNA transfer systems comes from the elegant experiments of Buchanan-Wollaston *et al.* (12). These workers demonstrated that in an *Agrobacterium* strain harboring a disarmed Ti plasmid (a Ti plasmid containing the *vir* genes but not the T-DNA or its borders), the conjugal origin of transfer sequence (*oriT*) and mobilization (*mob*) genes of the small wide-host-range plasmid RSF1010 can functionally replace T-DNA borders and generate an RSF1010 intermediate transferable to plants by the *vir* gene transfer machinery. This strongly supports a conjugal model of T-DNA transfer and further suggests that at least some of the Ti plasmid *vir* gene products are functionally equivalent to the transfer (*tra*) gene products of conjugal plasmids. With the *Escherichia coli* conjugative F plasmid, at least 15 plasmid *tra* gene products are involved in the assembly of a pilus structure used to initiate binding to the recipient cell and in the formation of a proposed membrane-spanning ssDNA transfer channel at the pilus base (13). In the *Agrobacterium* plant transformation process, however, the mechanism by which the T-DNA is transported across both the bacterial and plant cell membranes and walls is poorly understood. Attachment of *Agrobacterium* to plant cells is mediated by bacterial chromosomal gene products (14–16), and extracellular pili have not been implicated in virulence (2). By analogy with plasmid conjugal systems, however, T-complex export might likely be mediated by a *vir*-specified membrane-pore structure.

Products of the Ti plasmid *virB* operon are the best candidates to form a membrane-associated T-DNA transport apparatus. This hypothesis is based in part on the membrane localization of the 11 *virB* gene products predicted by the *virB* DNA sequence analysis (17–21) and confirmed in the case of several VirB proteins by cellular fractionation experiments (22–24). In addition, recent genetic studies have shown that at least three *virB* genes, *virB9*, *virB10*, and *virB11*, are essential for tumorigenicity (25). Because *virB* functions are not necessary for T-DNA processing reactions (26), this finding suggests that *virB* gene products mediate a late step (or steps) in the T-DNA transfer process. Interestingly, the predicted *virB11* gene product shares amino acid sequence similarity with ComG 1, a protein required for the uptake and passage of DNA through the cell wall of competent *Bacillis subtilis* cells (23, 27). Finally, the *virB10* gene product was recently identified in *A. tumefaciens* as part of an inner membrane protein aggregate (24), suggesting that VirB10 could be a component of a T-DNA transport apparatus. These data are all consistent with the proposed role of the *virB* gene products in mediating T-DNA transfer through the *Agrobacterium* envelope. In the present study we report that coordinate overexpression of the *virB9*, *virB10*, and *virB11* genes increases the efficiency of T-DNA transfer to plants in comparison with that of an RSF1010 plant transformation vector. Our results suggest that VirB functions affect the substrate specificity of the T-DNA transfer machinery,

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Abbreviations: T-DNA, transfer DNA; Ti plasmid, tumor-inducing plasmid; ssDNA, single-stranded DNA.

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strongly supporting the hypothesis that *virB* gene products mediate T-DNA transport to plants by *Agrobacterium*.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases and T4 DNA ligase were purchased from International Biotechnologies. The antibiotics geneticin (G418), carbenicillin, kanamycin, tetracycline, and vancomycin were obtained from Sigma. Cefotaxime was purchased from Calbiochem.

**Plasmids.** The plasmids used in this study are listed in Table 1. An RSF1010 plant transformation vector was constructed by inserting a 1.5-kilobase-pair (kb) *Eco*RI fragment containing the chimeric nopaline synthase–neomycin phosphotransferase II gene (*nptII*) from plasmid pEND4K (28) into the unique *Eco*RI site of the IncQ vector pKT231 (29). The resulting 14.5-kb plasmid, pJW323, contained all of the replication and *mob* functions of RSF1010, the *nptII* gene for selection of G418-resistant plant cells, and markers for the selection of kanamycin and streptomycin resistance in bacteria. The broad-host-range *virB* promoter cloning vectors pED8 and pED32, and the *virB* expression plasmids pED9, pED10, and pED11 have all been described (25). A 2.2-kb *Eco*RI fragment carrying the *virB9* and *virB10* genes was isolated from plasmid pJW327 (24) and inserted into the *Eco*RI site of plasmid pED8 (25), thereby placing these *virB* genes under transcriptional control of the *virB* promoter on the resulting plasmid pED35 (Table 1). The individual *virB9* gene was placed under *virB* promoter control by inserting a 2.1-kb *Hin*dIII–*Pst* I fragment from pED9 (25) into the corresponding restriction sites of the *virB* promoter expression vector pED32 (25) to yield pED37 (Table 1). All plasmids were mobilized from *E. coli* strain HB101 (31) into *A. tumefaciens* by triparental mating (32) with pRK2013 as the helper plasmid. All media, conditions for bacterial growth, and antibiotic concentrations for the selection of plasmids in *A. tumefaciens* and *E. coli* have been described (7, 25). The procedures for plasmid DNA isolation and manipulation were as described by Sambrook *et al.* (33).

**Leaf Transformations.** *Nicotiana tabacum* L. cv. Wisc. 38 leaf disc transformation was carried out as described (34). After 48 hr of cocultivation, leaf pieces were transferred to LS medium (35) without hormones (tumorigenesis assay) or LS containing 2 μg of α-naphthalene acetic acid (NAA), 0.3 μg of kinetin, and 10 μg of G418 per ml (G418 resistance assay); 200 μg of vancomycin and 500 μg of carbenicillin or 500 μg of cefotaxime (for strains carrying Tn3HoHo1) per ml were included to eliminate the bacteria.

## RESULTS

**Plant Transformation by the RSF1010 Vector pJW323.** We constructed an RSF1010-based plant transformation vector by inserting a chimeric nopaline synthase–neomycin phosphotransferase II (*nptII*) gene into the IncQ replicon pKT231 (29). The resulting 14.5-kb plasmid, pJW323, contained all of the replication and mobilization (*oriT*/*mob*) functions of RSF1010 but lacked T-DNA border sequences. Plasmid pJW323 was mobilized by triparental mating into the isogenic *A. tumefaciens* strains A136 (no Ti plasmid) (36) and A348 (pTiA6) and into the disarmed strain LBA4404 (37). The IncP, Mob$^-$ binary vector pEND4K (28), which contains an *nptII* gene flanked by the left and right borders from pTiA6, was also introduced into these strains to serve as a positive control for plant transformation. Plasmid pEND4K is based on the RK2 deletion derivative pTJS75-kan (28) and contains the RK2 *oriT* sequence but lacks several of the *mob* genes necessary for self-mobilization (38). The cognate missing *mob* functions are normally supplied by helper plasmids such as pRK2013 during the triparental mating procedure. A *Nicotiana tabacum* leaf disk assay showed that strain A348 delivered both pJW323 and pEND4K into plant cells as ascertained by transformation to G418 resistance (Table 2). Interestingly, pJW323 was delivered to plants at significantly lower frequencies than pEND4K when these vectors were in A348 but at similar frequencies when they were in LBA4404 (Table 2).

***virB9*, *virB10*, and *virB11* Are Required for Transfer of pJW323 to Plant Cells.** Previous complementation analysis of *A. tumefaciens virB* mutants demonstrated a requirement for the *virB9*, *virB10*, and *virB11* genes in T-DNA transfer (25). A similar strategy was employed in the present study to determine whether these three *virB* genes were also needed for the transfer of pJW323 to plants. Briefly, several wide-host-range *vir* promoter expression vectors (pED plasmids) (25) were used to express different combinations of the *virB9*, *virB10*, and *virB11* genes in trans in *Agrobacterium* mutants harboring polar Tn*3*HoHo1 (40) insertions in the *virB* region of the pTiA6 plasmid (see *Materials and Methods* and Table 1). In this way we analyzed strains missing only the *virB9*

Table 1. Bacterial strains and plasmids

| Plasmids | Characteristics | Ref. |
|---|---|---|
| pEND4K | Kan$^r$, Cam$^r$, broad-host-range IncP Mob$^-$/border$^+$ binary plant transformation vector containing an *nptII* plant selectable marker | 28 |
| pKT231 | Kan$^r$, Sm$^r$, broad-host-range IncQ Mob$^+$ plasmid containing a unique *Eco*RI cloning site | 29 |
| pJW323 | Kan$^r$, Sm$^r$, IncQ Mob$^+$/border$^-$ plasmid containing the *nptII* gene from pEND4K inserted as a 1.5-kb *Eco*RI fragment into pKT231 | This study |
| pJW327 | Carb$^r$, 2.2-kb *Eco*RI fragment containing *virB9* and *virB10* in pUC119 | 24 |
| pTJS75-tet | Tet$^r$, broad-host-range IncP plasmid with a unique *Hin*dIII cloning site | 30 |
| pED8 | Tet$^r$, pTJS75-tet containing the *virB* promoter on a 0.53-kb *Hin*dIII fragment | 25 |
| pED9 | Tet$^r$, contains the *virB9*, *virB10*, and *virB11* genes under control of the *virB* promoter in pTJS75-tet | 25 |
| pED10 | Tet$^r$, contains the *virB10* and *virB11* genes under control of the *virB* promoter in pTJS75-tet | 25 |
| pED11 | Tet$^r$, contains the *virB11* gene under control of the *virB* promoter in pTJS75-tet | 25 |
| pED32 | Tet$^r$, 0.6-kb *Hin*dIII fragment containing a unique *Eco*RI cloning site behind the *virB* promoter in pTJS75-tet | 25 |
| pED35 | Tet$^r$, 2.2-kb *Eco*RI fragment from pJW327 inserted into pED32 to place the *virB9* and *virB10* genes under control of the *virB* promoter | This study |
| pED37 | Tet$^r$, *Pst* I deletion derivative of pED9 containing *virB9* under control of the *virB* promoter | This study |

Kan$^r$, Cam$^r$, Sm$^r$, Carb$^r$, Tet$^r$, resistance to kanamycin, chloramphenicol, streptomycin, carbenicillin, and tetracycline.

Table 2. *N. tabacum* leaf disk transformation with pJW323 and pEND4K

| Strain | Bacteria,* no. per ml | Leaf pieces with G418$^r$ callus† | |
|---|---|---|---|
| | | % with at least one G418$^r$ callus† | % with at least two G418$^r$ calli |
| LBA4404 | | | |
| pKT231 | $10^9$ | 0 | 0 |
| pJW323 | $10^9$ | 100 | 96 |
| | $10^8$ | 96 | 89 |
| | $10^7$ | 100 | 97 |
| pEND4K | $10^9$ | 100 | 96 |
| | $10^8$ | 100 | 100 |
| | $10^7$ | 100 | 100 |
| A348 | | | |
| pKT231 | $10^9$ | 0 | 0 |
| pJW323 | $10^9$ | 40 | 11 |
| | $10^8$ | 31 | 7 |
| | $10^7$ | 8 | 1 |
| pEND4K | $10^9$ | 97 | 96 |
| | $10^8$ | 96 | 85 |
| | $10^7$ | 92 | 81 |

*A. *tumefaciens* cells containing a given plasmid were grown in MG/L broth (39) containing kanamycin at 10 μg per ml to an $OD_{600}$ ≈ 1.3. The cells were collected by centrifugation, resuspended, and serially diluted in LS plant growth medium (35) for use in cocultivation. Samples were diluted in sterile saline and plated on MG/L plates to determine viable counts.

†Bacterial cocultivation with tobacco leaf pieces and the selection of G418-resistant (G418$^r$) transformants was carried out as described. The results are presented as the percentage of leaf pieces displaying at least one or two G418$^r$ calli based on analysis of at least 32 leaf pieces per bacterial concentration and represent the average of two separate experiments.

(strain 26Mx pED10), *virB10* (strain 28Mx pED11), or *virB11* (strain 368Mx pED8) gene products for the ability to transfer pJW323. Table 3 shows that loss of any one of these three *virB* gene products completely abolished pJW323-mediated plant transformation to G418 resistance. However, when the missing *virB* functions were provided in trans, the ability of each of these strains to generate G418-resistant leaf calli was restored (Table 3). These results indicated that the *virB9*, *virB10*, and *virB11* gene products were required in *Agrobacterium* for plant transformation by pJW323. Further, based on the proposed model of *virB* function in the formation of a T-DNA transport channel, this suggested that both the T-complex and the pJW323 transfer intermediate (hereafter referred to as the R-complex) utilized the same *virB* transfer machinery.

**pJW323 Suppresses Virulence in *A. tumefaciens*.** The RSF1010 plant transformation experiments of Buchanan-Wollaston *et al.* (12) were performed with LBA4404, an *A. tumefaciens* strain containing a Ti plasmid lacking the T-DNA. For this reason, we were interested in determining whether the T-DNA border-independent transfer of pJW323 interfered with T-complex transfer. Both strains LBA4404 and A348 containing plasmid pEND4K transformed plant cells with a frequency similar to that obtained with strain LBA4404 pJW323 (Table 2). This suggested that the lower frequency of pJW323 transformation observed with strain A348 (Table 2) might not simply reflect an inherent difference in virulence between the two strains but instead could be due to the presence of T-DNA in the pTiA6 plasmid. Interestingly, virulence assays on *Kalanchoe daigremontiana* leaves revealed that pJW323 greatly suppressed T-DNA-mediated tumor formation by strain A348, whereas the presence of plasmid pEND4K had no affect on tumor induction (Table 4). Plasmid pKT231 (29), the IncQ parental vector of pJW323, also completely suppressed the virulence of strain A348 on *Kalanchoe* (data not presented). The observation that strain A348 generated G418-resistant leaf callus with pJW323 (Table 2) suggested that the oncogenic suppression caused by pJW323 was not due to a lack of bacterial–plant cell attachment. Further, virulence suppression probably did not result from a loss of the Ti plasmid or an inhibition of *vir* gene function by pJW323, since the work of Buchanan-Wollaston *et al.* (12, 41) and our results described above demonstrate that at least several of the *vir* loci are required for plant transformation by RSF1010. Rather, these data suggested that the T-complex and R-complex molecules might be acting as mutual antagonists either in the formation of transfer intermediates or in the transfer process itself.

**Overexpression of *virB9*, *virB10*, and *virB11* Specifically Enhances the Efficiency of T-DNA Transfer.** One possibility was that the pJW323 transfer intermediates were preventing T-complex transfer by saturating or jamming the VirB transport apparatus. This could be caused by formation of R-complex molecules in numerical excess of the T-complex, less efficient transport of the R-complex, or both. If so, we reasoned that pJW323-mediated virulence suppression might be overcome by increased expression of the *virB* gene products. In support of this idea, overproduction of the

Table 3. *N. tabacum* leaf disk transformation with pJW323 by *virB* mutant strains

| Strain | Missing *virB* gene | Bacteria,* no. per ml | Leaf pieces with G418$^r$ callus† | |
|---|---|---|---|---|
| | | | % with at least one G418$^r$ callus† | % with at least two G418$^r$ calli |
| A348 pJW323 pED8 | None | $10^9$ | 61 | 21 |
| | | $10^8$ | 27 | 7 |
| 26Mx pJW323 pED8 | *virB9, -10, -11* | $10^9$ | 0 | 0 |
| 26Mx pJW323 pED10 | *virB9* | $10^9$ | 0 | 0 |
| 26Mx pJW323 pED9 | None | $10^9$ | 33 | 7 |
| | | $10^8$ | 3 | 0 |
| 28Mx pJW323 pED8 | *virB10, -11* | $10^9$ | 0 | 0 |
| 28Mx pJW323 pED11 | *virB10* | $10^9$ | 0 | 0 |
| 28Mx pJW323 pED10 | None | $10^9$ | 84 | 56 |
| | | $10^8$ | 56 | 28 |
| 368Mx pJW323 pED8 | *virB11* | $10^9$ | 0 | 0 |
| 368Mx pJW323 pED11 | None | $10^9$ | 93 | 17 |
| | | $10^8$ | 37 | 17 |

*A. *tumefaciens* cells were prepared for cocultivation as described in the legend to Table 2 except that 4 μg of tetracycline per ml was also included in the growth medium of strains containing a pED plasmid.

†All procedures were as described in the legend to Table 2.

Table 4. Tumorigenicity of *A. tumefaciens* strain A348 containing various plasmids of *Kalanchoe* leaves

| Plasmid | Tumor formation* | *virB* genes overexpressed |
|---|---|---|
| None | ++++ | None |
| pEND4K | ++++ | None |
| pJW323 | − | None |
| pJW323, pED8 | − | None |
| pJW323, pED37 | − | *virB9* |
| pJW323, pED11 | − | *virB11* |
| pJW323, pED10 | − | *virB10, -11* |
| pJW323, pED35 | − | *virB9, -10* |
| pJW323, pED9 | +++ | *virB9, -10, -11* |

**Kalanchoe* leaves were scratched with an 18-gauge needle, and the wounds were inoculated with 5 μl of saline containing $5 \times 10^7$, $5 \times 10^6$, or $5 \times 10^5$ cells from a fresh overnight culture of strain A348 harboring the indicated plasmid(s). Tumor formation was scored after 28 days as follows: ++++, large tumors present at all inoculation sites; +++, large tumors present at inoculation sites of $5 \times 10^7$ and $5 \times 10^6$ cells but only small tumors present at inoculation site of $5 \times 10^5$ cells; −, no detectable tumors at any inoculation site.

VirB9, VirB10, and VirB11 proteins by plasmid pED9 (25) almost completely restored the tumorigenicity of strain A348 pJW323 on *Kalanchoe* leaves (Table 4). However, tumorigenicity was not restored by the parental expression vector pED8, by cooverexpression of *virB9* and *virB10* (pED35) or *virB10* and *virB11* (pED10), or by individual overexpression of the *virB9* (pED37) or *virB11* (pED11) genes (Table 4). The more quantitative *N. tabacum* leaf disk transformation assay revealed that pJW323 reduced the frequency of hormone-autonomous tumor formation (i.e., T-DNA transfer) by at least 10-fold in strain A348 (Fig. 1). Similar to the results obtained with *Kalanchoe*, experiments using tobacco leaf pieces demonstrated that plasmid pED9 also restored the tumorigenicity of A348 pJW323 back to the level of bacteria containing only plasmid pED8 (Fig. 1). Finally, although the presence of plasmid pED9 alone caused a detectable (≈50%) increase in tumorigenesis by A348 bacteria (Fig. 1) and resulted in a substantial (≈500%) increase in T-DNA-mediated tumorigenesis with strain A348 containing pJW323 (Fig. 1), the elevated *virB* gene expression did not increase the frequency of R-complex transfer to the level obtained with strain LBA4404 (Table 2 and Fig. 1).

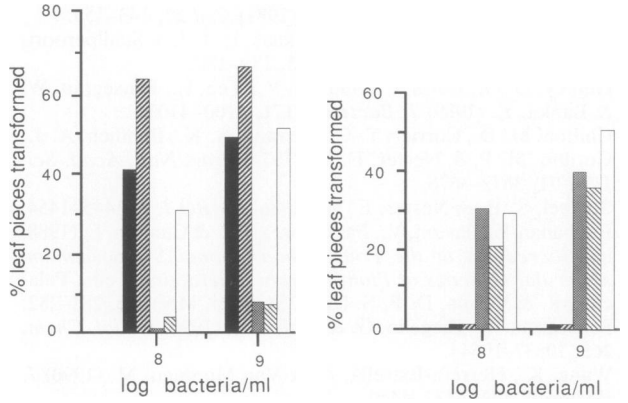


FIG. 1. Plant transformation to hormone autonomy (*Left*) and G418-resistance (*Right*) by *A. tumefaciens* strain A348 containing various plasmids. The procedures used for cocultivation of *Nicotiana tabacum* leaf pieces with *A. tumefaciens* cells and the selection of transformed leaf calli are described in *Materials and Methods*. At least 32 leaf pieces were cocultivated with each bacterial dilution. The results are presented as the percentage of leaf pieces displaying at least one transformed callus and represent the average of three separate experiments. ■, pED8; ▨, pED9; ▩, pJW323; ▧, pJW323 and pED8; □, pJW323 and pED9.

## DISCUSSION

We demonstrated previously that the *virB9*, *virB10*, and *virB11* gene products are essential for plant tumorigenesis (25). Here we show that these three VirB proteins are also required for the transfer of pJW323, a self-mobilizable IncQ plasmid, from *Agrobacterium* to plant cells (Table 3). Further, the presence of pJW323 greatly suppressed tumor formation in the pTiA6-containing *A. tumefaciens* strain A348. Surprisingly, pJW323-mediated oncogenic suppression was partially relieved by coordinate overexpression of the *virB9*, *virB10*, and *virB11* gene products (Table 4 and Fig. 1). These results strongly suggest that the VirB9, VirB10, and VirB11 proteins functionally interact in a manner dependent on their relative abundance to affect T-DNA transfer.

Several observations support the hypothesis that overexpression of VirB9, VirB10, and VirB11 restore oncogenicity in pJW323-containing bacteria by increasing the efficiency of DNA transfer. First, overexpression of these *virB* genes would not be expected to increase the copy number of the T-complex, because *virB* functions do not appear to be involved in T-DNA processing reactions (26). Second, the presence of plasmid pED9 did not significantly affect the frequency of pJW323 transfer to plants by strain A348 (Fig. 1), suggesting that the replication and *mob* functions of pJW323 were not inhibited. Further, plasmid pED9 in *A. tumefaciens* strain A348 increased tumor induction by ≈50% on tobacco leaf disks compared with A348 containing pED8 (Fig. 1). Thus, overproduction of VirB9, VirB10, and VirB11 appears to stimulate the process of DNA mobilization to plants by *Agrobacterium*. Perhaps these three VirB proteins are rate-limiting for assembly or activity of the transfer machinery.

Collectively, our data are consistent with a "competitive-inhibition" model of pJW323-mediated suppression of T-DNA transfer, which has the following features: (*i*) both T-complexes and R-complexes are transferred to plants through a VirB-transfer apparatus; (*ii*) in the absence of extra VirB9, VirB10, and VirB11 proteins, R-complexes saturate the DNA transfer apparatus and thereby prevent T-complex transfer; and (*iii*) overproduction of VirB9, VirB10, and VirB11 increases the overall efficiency of T-DNA transfer (or abundance of transfer machinery) in a fashion that reflects the substrate specificity of the transfer apparatus for T-complex mobilization. Assuming the R-complex level remains the same, they no longer saturate the transfer machinery and T-complex transfer is restored.

Overexpression of *virB9*, *virB10*, and *virB11* relieved pJW323-mediated suppression of tumor formation but did not significantly increase transfer of pJW323 as monitored by G418 resistance (Fig. 1). This suggests that the T-complex possesses physical characteristics or specific factors (e.g., Vir proteins) that result in more efficient transport of it, compared with the R-complex, in the presence of additional VirB9, VirB10, and VirB11 proteins. For example, instead of the VirD2 protein associated with the T-complex 5′ end (9–11), the R-complex most likely contains a 5′-Mob protein cap (42). Further, Buchanan-Wollaston *et al.* (41) have reported a reduced requirement for *virE2* function in RSF1010 transfer than for T-DNA transfer, suggesting that RSF1010 encodes its own SSB protein. The observation that the disarmed strain LBA4404 provides equivalent transfer of pJW323 compared with the true "T-DNA" of pEND4K (Table 2) suggests that T-DNA may out-compete R-DNA for *vir* components necessary for optimal DNA movement. Both the *virB9* and *virB10* gene products are membrane-associated (24, 25), and VirB10 has been identified as a component of an

inner membrane protein aggregate in *A. tumefaciens* (24), while VirB11 is an extrinsic membrane protein with potential protein kinase activity (23). Based on the present study, it is possible that these are involved in a specific VirD2–VirB or VirE2–VirB protein interaction necessary for targeting the T-DNA to the membrane localized transfer apparatus.

Interestingly, the Mob$^-$/border$^+$ IncP binary vector pEND4K was transferred to plant cells more efficiently than pJW323 by strain A348 (Table 2), and pEND4K did not suppress the tumorigenicity of strain A348 (Table 2). Based on Southern blot analysis, plasmids pEND4K and pJW323 display copy numbers of approximately 5 and 15, respectively, in strain A348 (data not presented), although the level of pEND4K and pJW323 transfer intermediates produced in *A. tumefaciens* is unknown. However, recent results indicating that T-DNA formation may be negatively regulated in *A. tumefaciens* (43) suggests that the pEND4K intermediate might not be made in excess of the T-complex. Further, it is likely that both the pEND4K and T-DNA transfer intermediates are structurally indistinguishable (i.e., contain VirD2 and VirE2) by the VirB transfer machinery, since both are derived from T-DNA border processing reactions. The RSF1010 competitive-inhibition model described above predicts that pJW323 should also reduce the plant transfer frequency of a Mob$^-$/border$^+$ binary vector like pEND4K, and this is supported by recent results from our laboratory (A.N.B., unpublished data). Finally, if pJW323 suppresses tumorigenicity through the formation of conjugal intermediates that inhibit T-complex transfer by the VirB transport apparatus, then other *oriT/mob* plasmids might be expected to attenuate *Agrobacterium* virulence in the same manner. One example of this might be the broad-host range, self-mobilizable IncW plasmid pSa, which has been reported to suppress the tumorigenicity of *A. tumefaciens* in a *mob* gene-dependent fashion (44).

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