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Osa Protein Constitutes a Strong Oncogenic Suppression System That Can Block *vir*-Dependent Transfer of IncQ Plasmids between *Agrobacterium* Cells and the Establishment of IncQ Plasmids in Plant Cells

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The *osa* (oncogenic suppressive activity) gene of the IncW group plasmid pSa is sufficient to suppress tumorigenesis by *Agrobacterium tumefaciens*. *osa* confers oncogenic suppression by inhibiting VirE2 protein export. This result is similar, but not identical, to that of oncogenic suppression by the IncQ plasmid RSF1010. We conducted a series of experiments to compare oncogenic suppression by these two systems. *Agrobacterium* strains harboring plasmids containing *osa* are more able to effect oncogenic suppression than are similar strains containing various RSF1010 derivatives. When *osa* is present within a donor *Agrobacterium* strain that also carries a derivative of RSF1010, the transfer of RSF1010 derivatives to recipient bacteria and their establishment in plants are blocked. Oncogenic suppression is still effected when the *osa* gene is integrated into the *Agrobacterium* chromosome, suggesting that it is the *osa* gene product that is active in suppression and that suppression does not require a protein-nucleic acid intermediate like that described for IncQ plasmids. Extracellular complementation experiments with tobacco leaf disks indicated that Osa blocks stable transfer of RSF1010 to plant cells by inhibiting transfer of VirE2, which is essential for the transfer of RSF1010 into plant cells, and not by inhibiting the actual transfer of RSF1010 itself. Our results suggest that Osa and RSF1010 cause oncogenic suppression by using different mechanisms.

When coresident with the Ti (tumor-inducing) plasmid in *Agrobacterium tumefaciens*, the IncW plasmid pSa can inhibit the genetic transformation of plant cells (11, 23). The *osa* (oncogenic suppressive activity) gene of pSa is sufficient to inhibit plant transformation (7, 9). This phenomenon, called oncogenic suppression, resembles fertility inhibition of conjugative plasmid transfer by plasmids of different incompatibility groups (16, 24, 27). A possible explanation for oncogenic suppression is that *osa* inhibits the transfer of the *Agrobacterium* transfer DNA (T-DNA) to plant cells. However, it was demonstrated recently that *osa* does not affect the transfer of T-DNA; rather, *osa* inhibits the export of the single-stranded DNA binding protein VirE2 (19).

The IncQ plasmid RSF1010 can also cause oncogenic suppression (4, 34). Although RSF1010 is not self-transmissible, other plasmids, including the Ti plasmid, can mobilize it between *Agrobacterium* cells (3, 8). The Ti plasmid can also mobilize RSF1010 to plant cells; this transfer depends upon the type IV secretion system encoded by the *virB*/*virD4* genes (6, 34). Thus, RSF1010 conjugative transfer between *Agrobacterium* cells resembles T-DNA transfer from *Agrobacterium* to plant cells. Stahl et al. (33) reported that an RSF1010 nucleic acid-protein conjugative intermediate is required for oncogenic suppression of *Agrobacterium*. They suggested that this intermediate may compete with the VirD2–T-strand complex and/or VirE2 for the VirB/D4 export apparatus. In accordance with this model, Ward et al. (34) showed that overexpression of *virB9*, *virB10*, and *virB11* from an octopine-type Ti plasmid could reverse oncogenic suppression by RSF1010.

Although *osa* causes oncogenic suppression by blocking VirE2 but not T-DNA transfer to plant cells, RSF1010 apparently inhibits both VirE2 export and (to a lesser extent) T-DNA export (4). In addition, oncogenic suppression by *osa* cannot be reversed by overexpression of the *virB9*, *virB10*, and *virB11* genes from the nopaline-type Ti plasmid pTiC58 (19). We therefore sought to determine whether there is a fundamental difference between the mechanisms of oncogenic suppression by RSF1010 and pSa or whether the differences previously noted were merely quantitative.

The lack of reversal of oncogenic suppression by *osa* when additional copies of *virB9*, *virB10*, and *virB11* are expressed in *Agrobacterium*, along with our finding that a conjugative intermediate is not required for oncogenic suppression by *osa*, suggests that a distinctive mechanism of oncogenic suppression is conferred by Osa.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 describes the various *A. tumefaciens* strains and plasmids used in this study. *A. tumefaciens* was grown at 30°C in either YEP rich medium or AB-sucrose minimal medium (21). When appropriate, the following antibiotics were used: rifampin (10 μg/ml), carbenicillin (100 μg/ml in solid medium and 50 μg/ml in liquid medium); kanamycin (25 μg/ml), spectinomycin (100 μg/ml), erythromycin (150 μg/ml), and tetracycline (2 μg/ml in liquid medium and 10 μg/ml in solid medium).

Tumorigenesis assays. *Kalanchöe daigremontiana* plants were used for tumorigenesis assays on leaves. *A. tumefaciens* cells were grown to the stationary phase, washed, and resuspended in 0.9% NaCl to cell densities of 10^9, 10^{10}, and 10^{11} cells/ml. Ten-microliter portions of each cell suspension were inoculated onto multiple wound sites on the leaves. Visible tumors developed approximately 2

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TABLE 1. *A. tumefaciens* strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristics	Antibiotic resistance[b]	Reference or source
Strains			
A136	Nononcogenic derivative of C58; lacks pTi	Rif	35
A208	Oncogenic nopaline-type strain; contains pTiT37	Rif	31
A281	Oncogenic agropine-type strain; contains pTiBo542	Rif	31
A348	Oncogenic octopine-type strain; contains pTiA6	Rif	14
At221	Tn*3*-HoHoI insertion in *virE2*; A348mx358	Car, Rif	32
At333	UIA143	Ery	12
At902	LBA4404(pUCD3960)	Rif, Spe	19
At903	LBA4404(pUCD5533)	Rif, Spe	19
At906	At221(pUCD3960)	Car, Spe	19
At907	At221(pUCD5533)	Car, Spe	19
At928	A208(pBISN1)	Kan, Rif	19
At931	A208(pBISN1, pUCD3960)	Car, Kan, Rif, Spe	19
At932	A208(pBISN1, pUCD5533)	Car, Kan, Rif, Spe	19
At986	A208(pBISN1, pJB31)	Kan, Spe	This study
At998	A136(pJW323)	Kan, Rif	This study
At999	A348(pJW323)	Kan, Rif	This study
At1000	A208(pJW323)	Kan, Rif	This study
At1001	A281(pJW323)	Kan, Rif	This study
At1002	A136(pUCD5533)	Car, Rif, Spe	This study
At1003	A348(pUCD5533)	Car, Rif, Spe	This study
At1004	A208(pUCD5533)	Car, Rif, Spe	This study
At1005	A281(pUCD5533)	Car, Rif, Spe	This study
At1006	A348(pJW323, pUCD5533)	Car, Kan, Rif, Spe	This study
At1007	A348(pJW323, pUCD3960)	Car, Kan, Rif, Spe	This study
At1008	A136(pUCD3960)	Car, Rif, Spe	This study
At1009	A348(pUCD3960)	Car, Rif, Spe	This study
At1011	A281(pUCD3960)	Car, Rif, Spe	This study
At1016	A136(pSa)	Chl, Kan, Rif, Spe, Suf	This study
At1017	A348(pSa)	Chl, Kan, Rif, Spe, Suf	This study
At1018	A208(pSa)	Chl, Kan, Rif, Spe, Suf	This study
At1019	A281(pSa)	Chl, Kan, Rif, Spe, Suf	This study
At1020	A136(pSa::*neo*)	Chl, Kan, Neo, Rif, Spe, Sul	This study
At1021	A348(pSa::*neo*)	Chl, Kan, Neo, Rif, Spe, Sul	This study
At1022	A208(pSa::*neo*)	Chl, Kan, Neo, Rif, Spe, Sul	This study
At1023	A281(pSa::*neo*)	Chl, Kan, Neo, Rif, Spe, Sul	This study
At1055	A348(pML122, pUCD3960)	Car, Gen, Kan, Rif, Spe	This study
At1056	A348(pML122, pUCD5533)	Car, Gen, Kan, Rif, Spe	This study
At1057	A136(pML122)	Gen, Kan, Rif	This study
At1058	A348(pML122)	Gen, Kan, Rif	This study
At1059	A208(pML122)	Gen, Kan, Rif	This study
At1060	A281(pML122)	Gen, Kan, Rif	This study
At1076	A136(pJB31)	Rif, Spe	This study
At1077	A348(pJB31)	Rif, Spe	This study
At1078	A208(pJB31)	Rif, Spe	This study
At1079	A281(pJB31)	Rif, Spe	This study
At1080	A281::*osa*	Rif, Spe	This study
At1081	A348::vector	Rif, Spe	This study
At1084	A348::*osa*	Rif, Spe	This study
At1096	A348Δ*virB*	Rif	1
At1111	At1096::*osa*	Rif, Spe	This study
At1112	At1096::vector	Rif, Spe	This study
At1113	A208(pBISN1, pED9)	Kan, Rif, Tet	This study
At1114	A208(pBISN1, pED9, pUCD3960)	Car, Kan, Rif, Spe, Tet	This study
At1115	A208(pBISN1, pED9, pUCD5533)	Car, Kan, Rif, Spe, Tet	This study
At1116	A208(pBISN1, pED9, pJB31)	Kan, Rif, Spe, Tet	This study
At1148	At1081(pML122)	Gen, Kan, Rif, Spe	This study
At1149	At1084(pML122)	Gen, Kan, Rif, Spe	This study
At1160	UIA143(pTiA6)	Ery, Rif	5
At1181	UIA143(pTiA6, pE1649)	Ery, Spe, Tet	This study
At1182	UIA143(pTiA6, pE1650)	Ery, Spe, Tet	This study
At1312	At902(pJW323)	Kan, Rif, Spe	This study
At1313	At903(pJW323)	Kan, Rid, Spe	This study
At1314	At906(pJW323)	Car, Kan, Spe	This study
At1315	At907(pJW323)	Car, Kan, Spe	This study
At1368	LBA1251(pTiA6, pML122, pUCD3960)	Car, Gen, Rif, Spe, Tet,	This study
At1369	LBA1251(pTiA6, pML122, pUCD5533)	Car, Gen, Rif, Spe, Tet	This study
LBA1251	C58 chromosome, lacks pTi and pAtC58	Rif	Paul Hooykaas
LBA4404	Disarmed octopine-type strain; lacks T-DNA	Rif	28

Continued on following page

TABLE 1—*Continued*

Strain or plasmid[a]	Relevant characteristics	Antibiotic resistance[b]	Reference or source
Plasmids			
pAD1361	Complete *virB* operon plus *virGN54D*	Car, Tet	10
pBISN1	T-DNA binary vector; contains a *nos-nptII* and superpromoter-*gusA* intron gene	Kan	26
pJB31	RSF1010 derivative	Spe	2
pJW323	RSF1010 derivative, contains a *nos-nptII* plant-selectable marker	Kan	34
pML122	RSF1010-derived broad-host-range vector	Gen, Tet	18
pE578	IncP plasmid carrying the *pgl*/*picA* locus as an EcoRI fragment from the *A. tumefaciens* C58 chromosome	Tet	30
pE1649	PstI fragment containing the *osa* and spectinomycin resistance genes cloned into pE578	Spe, Tet	This study
pE1650	PstI fragment containing a spectinomycin resistance gene cloned into pE578; vector control for *osa* construction	Spe, Tet	This study
pED9	*virB*, *virB10*, and *virB11* genes under the control of the pTiA6 *virB* promoter	Tet	34
pPH1JI	IncP plasmid; used for eviction of other IncP plasmids during marker exchange mutagenesis	Gen	15
pSa	Oncogenic suppressive plasmid	Chl, Kan, Spe, Sul	23
pSa::*neo*	Neomycin phosphotransferase gene disruption of the *osa* gene of pSa	Chl, Kan, Neo, Spe, Sul	19
pUCD3960	*osa* gene under the control of an *npt* promoter in pUCD105	Car, Spe	19
pUCD5533	Asp718-blunted derivative of pUCD105	Car, Spe	19

[a] Laboratory stock designations: A, E. W. Nester; At, S. B. Gelvin; UCD, C. I. Kado; AD, A. Das.
[b] Car, carbenicillin; Chl, chloramphenicol; Ery, erythromycin; Gen, gentamicin; Kan, kanamycin; Neo, neomycin; Rif, rifampicin; Spe, spectinomycin; Sul, sulfonamide; Tet, tetracycline.

weeks after inoculation, and well-developed tumors were photographed approximately 4 weeks after inoculation.

Tobacco leaf disk assays. Disks from axenically grown *Nicotiana tabacum* cv. Wisconsin 38 were cut with a cork borer and inoculated with *A. tumefaciens* as previously described (19). Two days after inoculation, disks were moved to selective medium containing 100 μg of timentin per ml to kill *Agrobacterium*. To select for tumors, disks were incubated on MS medium (25) lacking phytohormones. To select for kanamycin-resistant calli, disks were incubated on MS medium containing phytohormones (callus-inducing medium) and 100 μg of kanamycin per ml. To assay for β-glucuronidase activity, disks were sampled, stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) overnight, and then destained in 70% ethanol (17). For extracellular complementation experiments, equal concentrations of bacteria (RSF1010 donor or VirE2 donor) were mixed and used to infect tobacco leaf disks for 2 days. Leaf disks were then transferred to callus-inducing medium containing timentin to kill *Agrobacterium* and kanamycin to select for kanamycin-resistant calli.

Bacterial conjugation. Octopine-type *Agrobacterium* strain A348 (At6) containing a kanamycin-resistant derivative of RSF1010, pML122 (provided by Eugene Nester), with or without an *osa* gene construction was used as a donor. As mentioned below, strain A348 with or without the *osa* gene integrated into the chromosomal *pgl*/*picA* locus was also used as a donor. pTi-cured nopaline-type *Agrobacterium* strain UIA143 (At333, erythromycin resistant) with or without pTiA6 and with or without *osa* was used as a recipient. Both donor and recipient cells were grown at 30°C in YEP medium overnight. The next day, cells were transferred to glucose-containing induction medium (pH 5.6) with or without 100 μM acetosyringone to obtain a density of 2.5×10^8 cells/ml. Cells were grown at 22°C for 6 h. Donor and recipient cells were mixed at a ratio of 7:1, and 10 μl of the cell mixture was spotted onto AB glucose agar induction medium (pH 5.6) containing or lacking 100 μM acetosyringone. The conjugation plates were incubated at 20°C for 3 days. One milliliter of 0.9% NaCl was used to wash and resuspend the conjugation mixture from the plates. Tenfold serial dilutions were made in 0.9% NaCl, and 100-μl portions of appropriate dilutions were plated on AB-sucrose medium containing both erythromycin and kanamycin to select for transconjugants that harbored pML122. Donor and recipient cells were quantified by plating the bacteria on AB-sucrose medium containing rifampin and on AB-sucrose medium containing erythromycin, respectively.

Chromosomal integration of the *osa* gene. A PstI fragment containing both *osa* and the spectinomycin resistance gene from pUCD3960 (7) or a control fragment lacking the *osa* gene from pUCD5533 (19) was cloned into the PstI site of plasmid pE578 between the *pgl* and *picA* genes of *A. tumefaciens* (20), generating pE1649 and pE1650, respectively. Plasmids pE1649 and pE1650 were electroporated into various *Agrobacterium* strains (A136, A348, A208, and A281). Spectinomycin- and tetracycline-resistant transformants were selected. Subsequently, plasmid pPH1JI (gentamicin resistant) was introduced by conjugation into each of these *Agrobacterium* strains, and transconjugants which were resistant to gentamicin and spectinomycin and sensitive to tetracycline were selected. Total DNA from the colonies was isolated and subjected to EcoRI digestion and gel electrophoresis and blotted onto nylon membranes. A 3.1-kbp EcoRI fragment containing the *pgl*/*picA* locus was used as a hybridization probe to confirm integration of the *osa* gene into the *Agrobacterium* chromosome.

RESULTS

***A. tumefaciens* strains containing the IncW plasmid pSa show stronger oncogenic suppression than strains containing many derivatives of the IncQ plasmid RSF1010 show when they are inoculated onto *Kalanchöe* plants.** Both the *osa* gene and the IncQ plasmid RSF1010 confer oncogenic suppression upon *Agrobacterium*. We therefore tested several strains of *Agrobacterium* harboring various pSa or RSF1010 derivatives to determine the strength of oncogenic suppression conferred by IncW and IncQ plasmids. *Agrobacterium* strains A136 (lacking a Ti plasmid), A208 (containing the nopaline-type Ti plasmid pTiT37), A348 (containing the octopine-type Ti plasmid pTiA6), and A281 (containing the supervirulent agropine-type Ti plasmid pTiBo542) were used to test oncogenic suppression on leaves of *Kalanchoë* plants by the IncW plasmid pSa, a plasmid carrying the *osa* gene (pUCD3960), or derivatives of

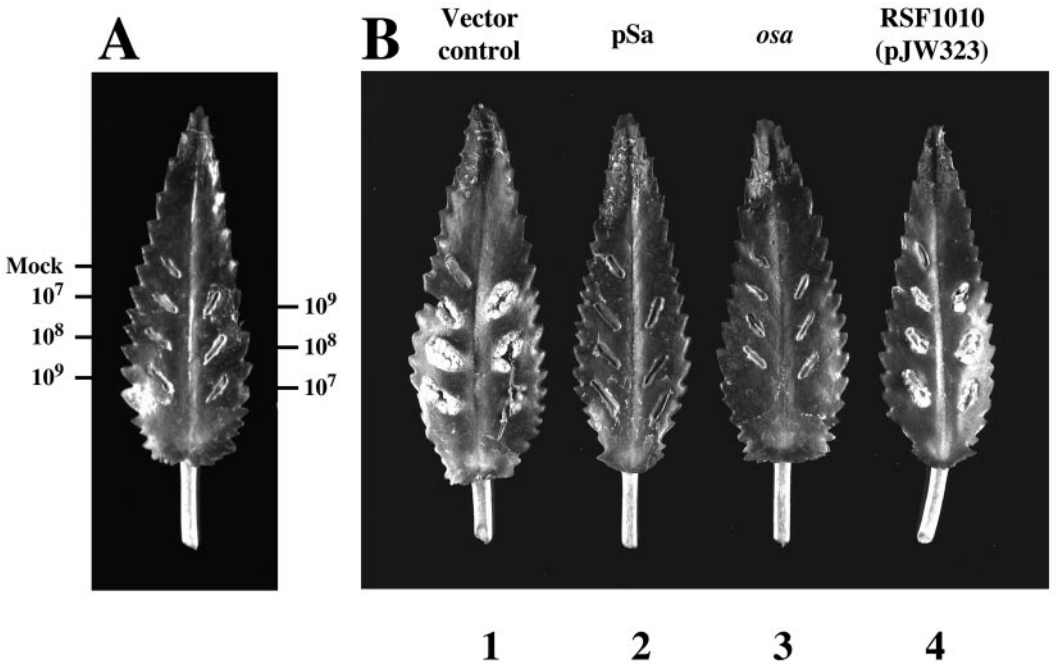

FIG. 1. Oncogenic suppression of *A. tumefaciens* strains by pSa, *osa*, and RSF1010. (A) Template for inoculation of *Kalanchoë* leaf wound sites. The number of bacteria inoculated into each wound is indicated. (B) Inoculation with *A. tumefaciens* A348 containing plasmids. Leaf 1, At1003; leaf 2, At1017; leaf 3, At1009; leaf 4, At999. The leaves were photographed 1 month after inoculation.

the IncQ plasmid RSF1010 (pML122, pJB31, and pJW323). Figure 1 shows the results of oncogenic suppression of *A. tumefaciens* A348 by pSa, *osa*, and pJW323. *osa* either on its parental plasmid pSa or on the pTAR-based plasmid pUCD3960 was more complete in oncogenic suppression than the RSF1010 derivatives pJB31 and pJW323 were. Similar results were obtained with *A. tumefaciens* A208 and A281 (data not shown). When the *osa* gene was disrupted by a neomycin cassette (pSa::*neo*), no oncogenic suppression was observed (data not shown). These results indicate that *Agrobacterium* strains harboring pSa or the *osa* gene are less virulent than *Agrobacterium* strains harboring various derivatives of RSF1010 are and that therefore *osa* may be a more potent inhibitor of virulence than RSF1010 is.

***osa* inhibits the establishment of RSF1010 in plant cells by inhibiting the transfer of VirE2 protein.** *A. tumefaciens* can transfer the RSF1010 derivative pJW323, carrying a plant-active *nptII* gene, to plant cells. Although pJW323 partially inhibits tumorigenesis by *A. tumefaciens*, the infected plant cells may become kanamycin resistant as a result of stable expression of the *nptII* gene (34). We introduced pJW323 into *A. tumefaciens* strains containing either *osa* on plasmid pUCD3960 or the vector control plasmid pUCD5533 and infected tobacco leaf disks to determine whether *osa* inhibits the transfer of RSF1010 from *A. tumefaciens* to plant cells. The presence of *osa*, but not the presence of the vector control plasmid, in *A. tumefaciens* suppressed formation of both tumors and kanamycin-resistant calli (Fig. 2). When we infected tobacco leaf disks with an *A. tumefaciens* strain containing pJW323 plus the vector control plasmid, some small tumors still developed on disks incubated on plant medium lacking phytohormones (Fig. 2A). As a result of transfer of pJW323, some kanamycin-resistant calli developed on kanamycin-containing callus-inducing medium (Fig. 2B). These data are consistent with our findings that the presence of pJW323 in *A. tumefaciens* only partially inhibits tumorigenesis on *Kalanchöe* (Fig. 1) and further indicate that *osa* can inhibit the establishment of RSF1010 in plant cells.

We next introduced pJW323 into *virE2* mutant and VirE2 donor *Agrobacterium* strains harboring either *osa* or a vector control plasmid and infected tobacco leaf disks with various combinations of *Agrobacterium* strains in extracellular complementation experiments. Two days after cocultivation with various bacterial combinations, leaf disks were transferred to either plant basal medium lacking phytohormones to detect tumor formation (data not shown) or callus-inducing medium containing kanamycin to detect the formation of kanamycin-resistant calli as a result of RSF1010 transfer. The results of experiments to investigate kanamycin-resistant callus formation are shown in Fig. 3. *A. tumefaciens* A348 containing pJW323 (At999) alone was able to transfer RSF1010 into plant cells to form kanamycin-resistant calli on 59% of the leaf disks (Fig. 3A and B). The VirE2 donor strain LBA4404 (At1313), but not *virE2* mutant strain At1314 or At1315 containing RSF1010, also incited kanamycin-resistant calli (Fig. 3C and D) (38% of the leaf disks with At1313 compared with no leaf disks with At1314 or At1315). These results indicate that VirE2 is required for establishment of RSF1010 in plant cells, as previously described (4). The presence of *osa* in strain At1312 completely blocked the formation of kanamycin-resistant calli. Kanamycin-resistant callus formation could be restored by coinfecting leaf disks with a mixture of a VirE2 donor (At903) and either At1314 (64% of the disks) or At1315 (29% of the disks) (the At903 strain provided VirE2 and the At1314 and At1315 strains provided RSF1010) (Fig. 3G and H). On the other hand, when VirE2 export was blocked by *osa* in *A.*

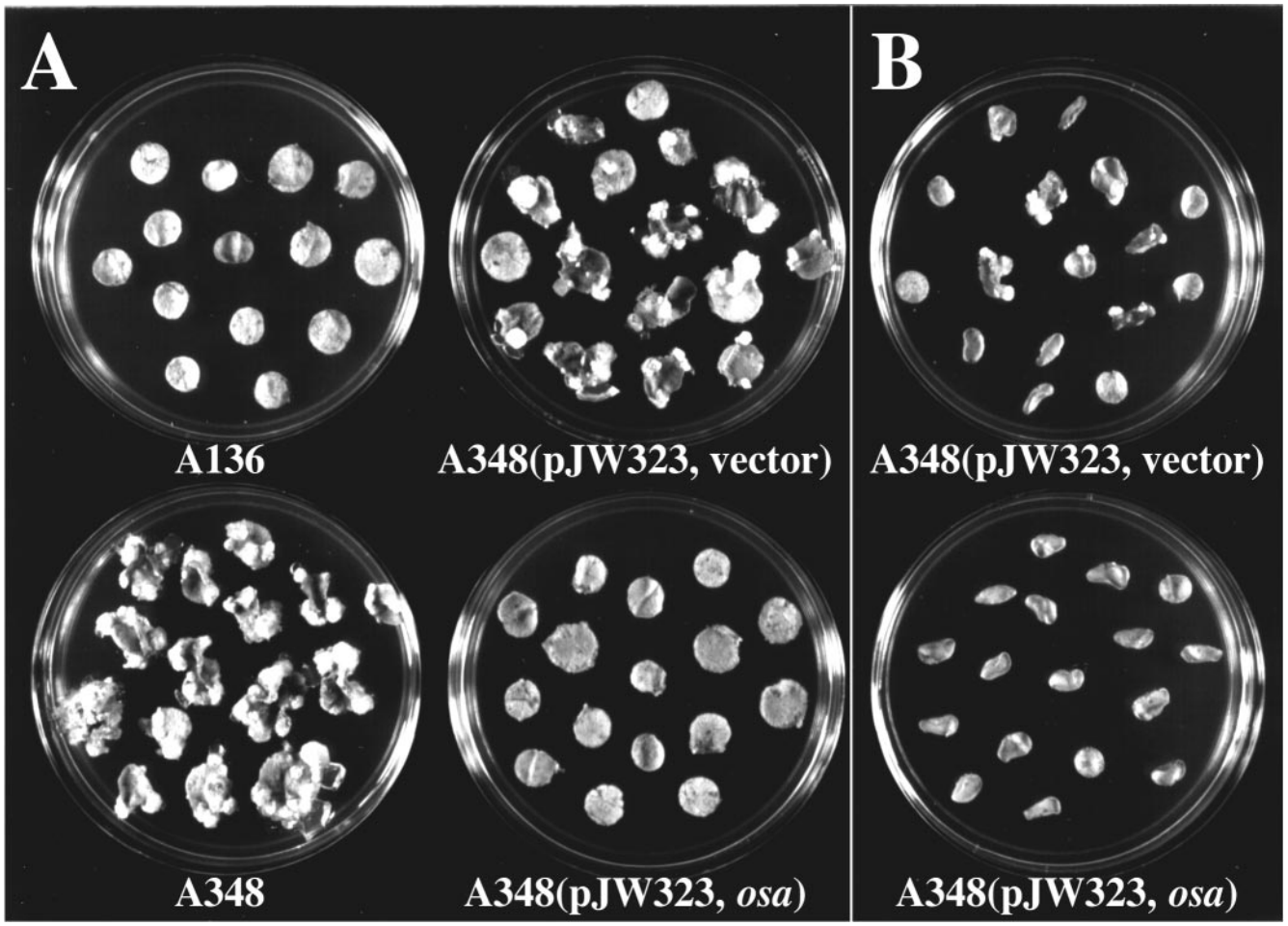

FIG. 2. *osa* inhibits transformation of tobacco leaf disks by RSF1010. Tobacco leaf disks were inoculated with various *A. tumefaciens* strains for 2 days and then were transferred to MS medium containing timentin and either lacking phytohormones (A) or containing phytohormones and kanamycin (B). The plates were photographed 1 month after infection. A136, no pTi; A348, A136 containing pTiA6; A348(pJW323, vector), At1006; A348(pJW323, *osa*), At1007.

tumefaciens At902, neither At1313 nor At1314 induced formation of any kanamycin-resistant calli (Fig. 3E and F). Taken together, these results indicate that *osa* does not block RSF1010 transfer per se to plant cells but rather inhibits VirE2 transfer that is required for formation of kanamycin-resistant calli after RSF1010 transfer.

***osa* inhibits *virB*-mediated conjugative transfer of RSF1010 between *Agrobacterium* cells.** *A. tumefaciens* can transfer RSF1010 derivatives between bacterial cells in a *virB*-dependent manner (3). To determine whether *osa* inhibits conjugative transfer of RSF1010 between *Agrobacterium* cells, we introduced pML122 into the rifampin-resistant strain *A. tumefaciens* A348. pML122 is a derivative of RSF1010 that contains a kanamycin resistance marker (18). We also introduced into this strain either a plasmid that contains *osa* or a control plasmid and mated the resulting strains (At1055 and At1056, respectively) with the erythromycin-resistant strain UIA143, selecting for kanamycin- and erythromycin-resistant bacteria. Table 2 shows that the conjugative transfer of pML122 depended upon the *vir* gene inducing the compound acetosyringone, as previously reported (3, 13). Conjugative transfer of pML122 was markedly decreased (by 4 orders of magnitude) when *osa* was present in the *Agrobacterium* donor strain.

To determine whether *osa* can function in the recipient bacterial cell to inhibit the conjugative transfer of RSF1010, we introduced into *A. tumefaciens* UIA143(pTiA6) a plasmid carrying *osa* or an empty vector control plasmid and used these strains as recipients in mating experiments. Our results indicate that although *osa* could inhibit the acetosyrongone-dependent transfer of RSF1010 when it was present in donor cells, it did not inhibit conjugative transfer when it was present in the recipient cells (data not shown).

A conjugative intermediate is not required for oncogenic suppression and inhibition of RSF1010 transfer between bacteria by *osa*. To date, all evidence for the oncogenic suppressive effect of *osa* and the inhibition of RSF1010 conjugative transfer by *osa* has involved *A. tumefaciens* strains harboring *osa* on a plasmid. Stahl et al. (33) reported that a nucleic acid-protein conjugative intermediate of RSF1010 is required for the oncogenic suppressive effect of RSF1010. In order to determine whether oncogenic suppression by *osa* requires a plasmid DNA-Osa protein complex as a conjugative interme-

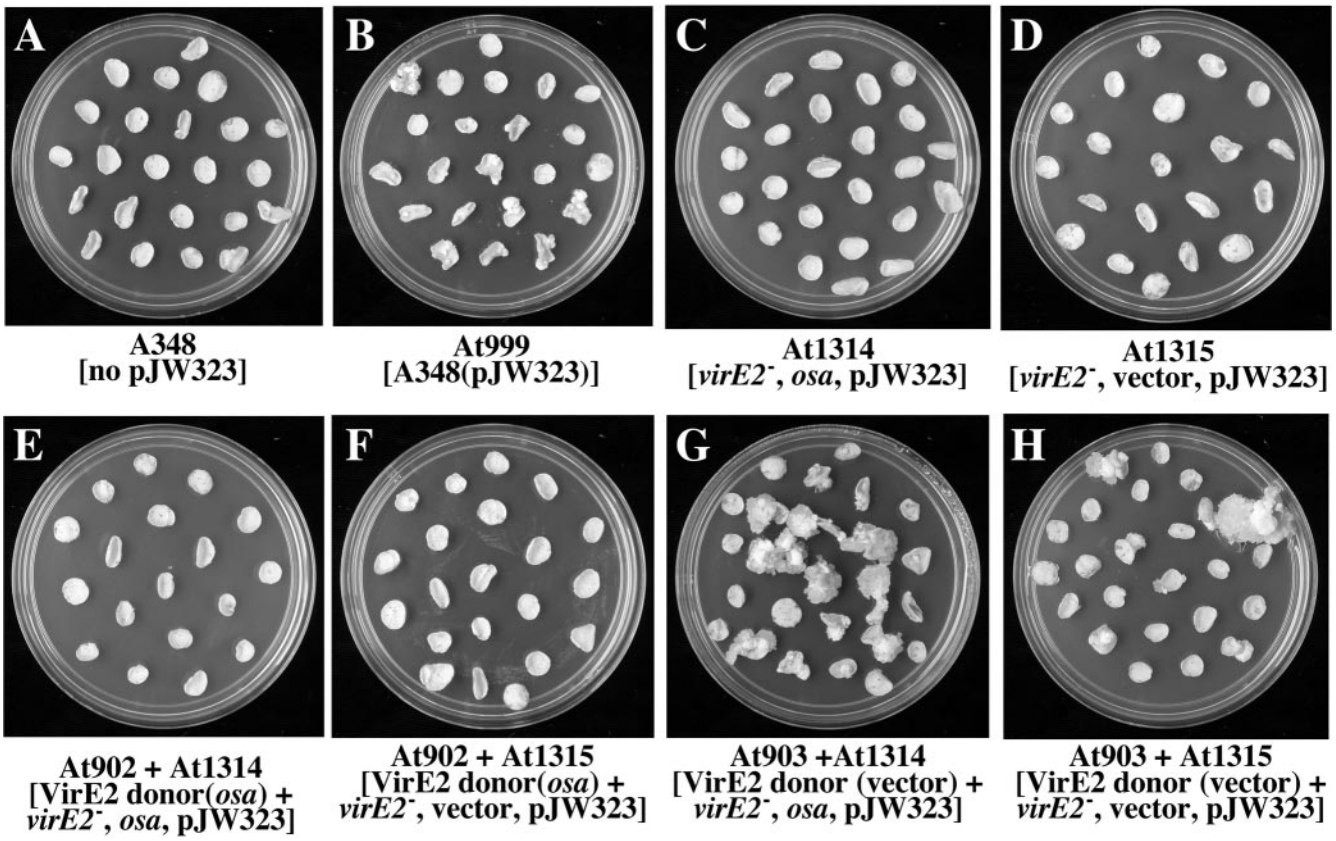

FIG. 3. *osa* blocks RSF1010 transfer from *A. tumefaciens* to plants by inhibiting the transfer of VirE2. Tobacco leaf disks were inoculated with various *A. tumefaciens* strains for 2 days and then were transferred to MS medium containing timentin, phytohormones, and kanamycin. The plates were photographed 1 month after infection.

diate, we constructed *A. tumefaciens* strains containing the *osa* gene inserted into the *pgl*/*picA* locus (31) of the *A. tumefaciens* C58 chromosome. Rong et al. (30) previously demonstrated that disruption of this chromosomal locus does not affect tumorigenesis. We used strains containing the *osa* gene either on a plasmid or integrated into the chromosome and vector control strains to inoculate wound sites on *Kalanchöe* leaves. Figure 4 shows that *osa* suppressed the oncogenicity of *A. tumefaciens* strains A348 and A281 regardless of whether the *osa* gene was located on a plasmid [A348(*osa*) and A281(*osa*)] or integrated into the chromosome [A348::*osa* (At1084) and A281::*osa* (At1080)]. The presence in these strains of an empty plasmid vector either as a separate replicon or integrated into the chromosome did not result in oncogenic suppression. These results suggest that expression of the *osa* gene from a chromosomal locus is sufficient to cause oncogenic suppression even of the highly oncogenic strain *A. tumefaciens* A281. The *A. tumefaciens* strains containing *osa* integrated into the chromosome do not contain a plasmid that is transfer competent under the conditions used. Because no transfer-competent relaxation complex can be formed in these strains, our results suggest that the Osa protein itself directly effects oncogenic suppression. It is highly unlikely that oncogenic suppression is mediated by *osa* mRNA because disruption of the Osa open reading frame results in loss of oncogenic suppression (data not shown).

TABLE 2. *osa* inhibits the *vir* gene-dependent conjugative transfer of RSF1010[a]

Strain	Acetosyringone induction	Transfer frequency (transconjugant/donor)
A348(pML122, vector) (At1056)	−	$<1.9 \times 10^{-12}$
	+	1.3×10^{-7}
A348(pML122, *osa*) (At1055)	−	$<2.2 \times 10^{-12}$
	+	$<4.1 \times 10^{-11}$

[a] *A. tumefaciens* A348 was used as the donor, and *A. tumefaciens* UIA143 was used as the recipient. The results are representative of five independent experiments.

We further tested whether the presence of *osa* within a chromosomal locus inhibits the conjugative transfer of RSF1010 between *Agrobacterium* cells. In these experiments, we used as a recipient an *A. tumefaciens* strain containing a Ti plasmid [UIA143(pTiA6) (= At1160)] because Bohne et al. (5) and Liu and Binns (22) showed that the use of such a strain increases the conjugation frequency of RSF1010 derivatives. Table 3 shows that when we used an *Agrobacterium* strain (At1149) carrying the *osa* gene integrated into the donor bacterial chromosome, conjugative transfer of RSF1010 (pML122) was inhibited to an extent similar to the extent

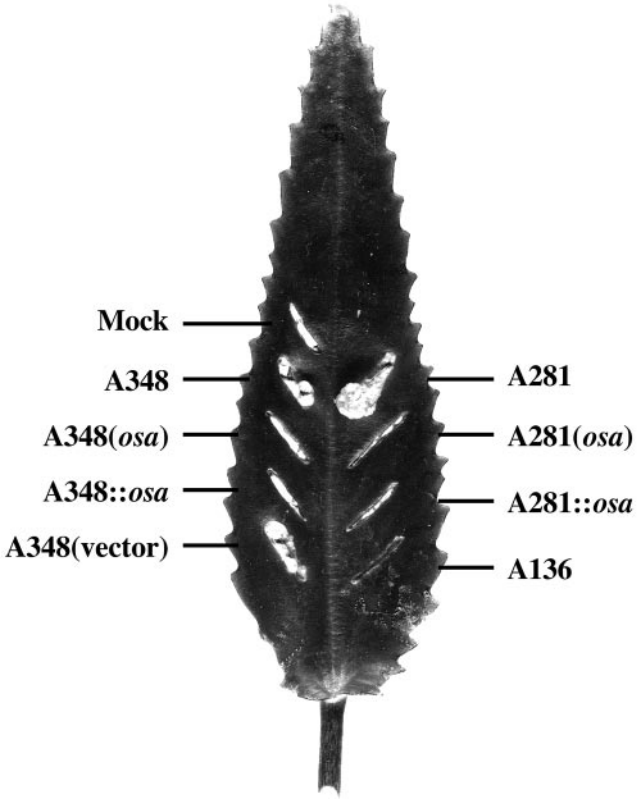

FIG. 4. *osa* can confer oncogenic suppression when it is integrated into the *A. tumefaciens* chromosome. Wound sites on a *Kalachöe* leaf were inoculated with 10^9 cells of various *A. tumefaciens* strains, and the leaf was photographed 1 month later. A348, A136 containing pTiA6; A281, A136 containing pTiBo542; A348(*osa*), At1009; A281(*osa*), At1011; A348::*osa*, At1084; A281::*osa*, At1080; A348(vector), At1081; A136, no pTi.

observed when *osa* was present on a plasmid in the donor bacterium (Table 2).

Conjugative transfer of RSF1010 between *Agrobacterium* cells is inhibited by *osa* in the absence of plasmid pAtC58. Many *A. tumefaciens* strains, including the strains used in the experiments described above, contain plasmid pAtC58. Chen et al. (8) showed that pAtC58 encodes a type IV secretion system, AvhB, that can promote the conjugative transfer of RSF1010 between *Agrobacterium* cells. We therefore sought to determine whether *osa* inhibited the conjugative transfer of RSF1010 in the absence of the AvhB transfer system.

TABLE 3. Inhibition of RSF1010 transfer by chromosome-localized *osa* genes[a]

Donor strain	Acetosyringone induction of donor	Conjugation frequency (per donor)	Conjugation frequency (per recipient)[b]
At1148 (A348::vector)	−	$<4.72 \times 10^{-10}$	$<3.05 \times 10^{-10}$
At1148 (A348::vector)	+	1.50×10^{-5}	9.95×10^{-6}
At1149 (A348::*osa*)	+	$<1.01 \times 10^{-9}$	$<3.83 \times 10^{-10}$

[a] The results are representative of three independent experiments.
[b] The recipient strain was *A. tumefaciens* UIA143(pTiA6) (At1160).

A. tumefaciens LBA1251 lacks both a Ti plasmid and pAtC58. We introduced into this strain pTiA6, the RSF1010 derivative pML122, and either pUCD3960 (containing *osa*) or pUCD5533 (empty vector control for the presence of *osa*), generating *A. tumefaciens* At1368 and At1369, respectively. Our results indicated that in the absence of the AvhB type IV secretion system encoded by pAtC58, *osa* inhibited conjugative transfer of RSF1010 by more than 4 orders of magnitude ($2.8 \times 10^{-5} \pm 0.6 \times 10^{-5}$/recipient for At1369; $<1 \times 10^{-9}$/recipient for At1368). Thus, *osa* inhibits RSF1010 transfer directed by the Ti plasmid.

DISCUSSION

Plasmids of the IncW group (such as pSa) and the IncQ group (such as RSF1010) can suppress tumorigenesis by *A. tumefaciens* on many plant species. The RSF1010 derivatives pJB31 and pJW323 were shown previously to suppress tumorigenesis by the octopine-type strain A348 on *N. tabacum* (tobacco) and *K. daigremontiana* (4, 33), whereas pSa inhibited transformation of numerous plant species by both strain C58 (7, 9, 11, 23) and strain A208 (19), which contain a nopaline-type Ti plasmid, and by strains A348 (19) and 1D1 (11), which contain an octopine-type Ti plasmid. Oncogenic suppression by pSa was more effective on *A. tumefaciens* C58 than suppression of strain 1D1 was (11).

In this study we investigated the relative strengths of derivatives of pSa and RSF1010 in conferring oncogenic suppression upon various *A. tumefaciens* strains. By using different numbers of *Agrobacterium* cells as inocula on *K. daigremontiana* leaves, we observed different extents of suppression of these oncogenic *Agrobacterium* strains. We concluded that the presence of pSa, or just the *osa* gene of pSa, elicits greater oncogenic suppression than the presence of several derivatives of RSF1010, including pJB31 and pJW323, elicits. Plasmid pSa is present at a level of 2 or 3 copies per bacterial cell (Lee and Gelvin, unpublished data), whereas the derivatives of RSF1010 are present at levels of ~20 copies per cell (4). Therefore, it is unlikely that the greater extent of oncogenic suppression effected by the *osa* gene than by the RSF1010 derivatives results from a higher copy number of the oncogenic suppressive entity.

The surprising finding of this study is that although oncogenic suppression of plant transformation by Osa involves inhibition of VirE2 but not T-DNA transfer, the effect of Osa on inhibition of RSF1010 conjugation may not involve VirE2. Previous studies indicated that mutation of *virE2* does not affect conjugative transfer of RSF1010 between *Agrobacterium* cells (13). However, these studies were performed with *Agrobacterium* strains that contained pAtC58. It is possible that pAtC58 can express some function equivalent to that of VirE2. If so, this function per se cannot be required for RSF1010 conjugative transfer, because elimination of pAtC58 from the bacterium still permitted conjugative transfer of RSF1010.

During *Agrobacterium*-mediated transformation of plants, VirE2 functions in the plant cell but probably not in the bacterium. A *virE2* mutant *Agrobacterium* strain can still deliver T-strands to a plant cell (36) and effect transformation if it is coinoculated with a VirE2-producing *Agrobacterium* strain (29) or if it is inoculated onto a transgenic plant that produces VirE2 (19). In the plant cell, VirE2 likely protects the T-strand

from nucleolytic degradation (36) and may be involved in targeting the T-complex to the plant nucleus (37). These functions would not be required for RSF1010 conjugative transfer between bacterial cells but would be required for RSF1010-mediated transformation of plants. Thus, inhibition of VirE2 export to plant cells could explain why Osa blocks plant transformation. The lack of importance of VirE2 in RSF1010 conjugative transfer would explain why Osa likely blocks conjugation by some process not involving VirE2.

Oncogenic suppression by Osa does not require the formation of a conjugative intermediate in the *Agrobacterium* cell. When we placed *osa* onto the *Agrobacterium* chromosome and eliminated any plasmid that could be transferred to the plant cell, oncogenic suppressive activity remained undiminished. This differs from the situation with RSF1010, where formation of a conjugative intermediate is required to effect oncogenic suppression (33). Therefore, although the consequences of the presence of the suppressive plasmids in the donor bacterial cells are the same, the suppressive mechanisms of the two systems likely differ. The Osa protein of the IncW plasmid pSa is sufficient to block the VirB/VirD4-mediated transfer of IncQ plasmid RSF1010 during bacterial conjugation. However, Osa does not block RSF1010 transfer from bacteria into plant cells. Rather, Osa blocks VirE2 export and subsequently the formation of kanamycin-resistant calli. Unlike RSF1010-mediated inhibition of T-DNA transfer, the inhibition of plant transformation by *osa* is a function of the Osa protein per se and is not associated with formation of a nucleic acid-protein complex. Thus, the oncogenic suppression by *osa* differs mechanistically from the suppression effected by RSF1010.

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