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Association of single-stranded transferred DNA from *Agrobacterium tumefaciens* with tobacco cells

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ABSTRACT **During the inception of crown gall tumorigenesis, the transferred DNA (T-DNA) is processed from the Ti (tumor inducing) plasmid of *Agrobacterium tumefaciens* and is transferred to plant cells. T-DNA processing and transfer require the induction of *vir* (virulence) genes by phenolic compounds secreted by wounded plant cells. After *vir* gene induction, both single-stranded (T-strands) and double-stranded forms of processed T-DNA accumulate in the bacteria. Although current models favor the transfer of T-strands to plants, there has yet been no experimental evidence to show this. In this paper, we show that T-strands disappear from acetosyringone-induced *A. tumefaciens* within 30 min of bacterial cocultivation with tobacco protoplasts. PCR analysis of T-DNA associated with protoplasts indicates that single-stranded, but not double-stranded, T-DNA can be detected in the plant cells within 30 min of bacterial cocultivation. Control experiments show that this T-DNA does not originate from lysed contaminating bacterial cells. T-DNA transfer depends on a functional bacterial *virB* operon. Protoplast infections using an *A. tumefaciens virE* mutant result in a low level of accumulation of T-strands in the plant cells.**

Crown gall disease results from transfer of a portion of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* to plant cells. This transferred DNA (T-DNA) integrates into plant nuclear DNA, where it directs synthesis of the phytohormones auxin and cytokinin and amino acid and sugar derivatives, termed opines (1, 2). Virulence (*vir*) genes residing on the non-T-DNA portion of the Ti plasmid direct the processing and transfer of T-DNA. Phenolic molecules such as acetosyringone induce the *vir* genes (3). An endonuclease encoded by the first two open reading frames of *virD* (VirD1 and VirD2) initiates T-DNA processing by nicking border repeat sequences that flank T-DNA (4–6). Single-stranded molecules of T-DNA (T-strands; ref. 7), associated with VirD2 protein covalently bound to the 5′ end (8–10) and coated with VirE2 single-stranded DNA binding protein (11, 12), accumulate in *A. tumefaciens* after incubation of the bacteria with acetosyringone. These "T-complexes" have been proposed as the form of T-DNA transferred to plant cells (13), most likely using bacterial membrane channels encoded by the *virB* operon (14–16). This model of T-DNA transfer to plant cells likens this process to the conjugal transfer of plasmids between bacterial cells (17). However, double-stranded processed T-DNA molecules also accumulate in acetosyringone-induced *A. tumefaciens* cells (18, 19). The form of T-DNA transferred to plant cells has, therefore, not yet been determined. We show here that *A. tumefaciens* transfers single-stranded DNA to the cytoplasm of tobacco cells within 30 min of cocultivation of the bacteria with regenerating tobacco protoplasts. This transfer depends on functional *virB* genes.

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MATERIALS AND METHODS

Bacterial Strains, Growth, and Induction Conditions. *A. tumefaciens* cultures were grown in AB minimal medium (20) containing 0.5% glucose, 25 μg of kanamycin per ml, and either 10 μg of rifampin or 50 μg of carbenicillin per ml. All *A. tumefaciens* strains harbored a multicopy plasmid consisting of *Sal* I fragment 13b (containing the *virG* gene of pTiA6) cloned into the *Sal* I site of pVK101 (21). This plasmid was mobilized from *Escherichia coli* by a triparental mating procedure (22) into *A. tumefaciens* A348(mx219) (*pinF*::Tn*3*-HoHo1) to create At493, *A. tumefaciens* A348(mx243) (*virB*::Tn*3*-HoHo1) to create At494, and *A. tumefaciens* A348(mx341) (*virE*::Tn*3*-HoHo1) to create At495 (23). For induction, bacterial cells were grown to a density of 2×10^9 cells per ml (Klett reading, 100; red filter), harvested by centrifugation at 12,000 × *g* for 10 min, and suspended in induction medium (19) containing 100 μM acetosyringone (for experiments investigating the loss of T-strands from the bacteria) or 2 μM acetosyringone (for experiments investigating the association of T-DNA with plant cells) at an initial concentration of 1×10^9 cells per ml. Bacteria were incubated at 25°C for 14 h in induction medium before use in the experiments described.

Southern Blot Analysis of DNA from Bacterial Cells. After *vir* gene induction by acetosyringone, bacteria were transferred to K3 medium (1×10^8 cells per ml) in the presence or absence of *Nicotiana tabacum* W38 protoplasts (24). After various periods of time, bacteria incubated in the presence of protoplasts were separated into bound and unbound fractions by filtration through Whatman no. 1 paper. The protoplast-bound bacteria were rinsed with cold K3 medium and washed from the filter with K3 medium. Protoplasts containing bound bacteria were collected by centrifugation for 5 min at 6000 × *g* and lysed by the addition of SDS to a final concentration of 1.0%. Bacteria bound to the plant cell walls were recovered by centrifugation at 6000 × *g* for 5 min. DNA was isolated from each bacterial sample by incubation of bacteria (≈10^7 cells in 0.4 ml) for 25 min at 37°C in 10 mM Tris·HCl, pH 7.0/1 mM EDTA/0.5% SDS/10 μg of RNase A per ml/1 mg of Pronase B per ml, following which the DNA was purified by phenol/chloroform extraction and precipitated with ethanol. DNA samples (1 μg, as measured fluorometrically using diaminobenzoic acid) were subjected to electrophoresis through 0.65% agarose gels and transferred to a nylon membrane using denaturing conditions; the membrane was hybridized with ^{32}P-labeled *Hin*dIII fragment 18c from the T-DNA (25). Hybridization was detected by autoradiography with Kodak XR-5 x-ray film.

Abbreviation: T-DNA, transferred DNA.

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PCR Analysis of T-DNA Associated with Tobacco Protoplasts. *A. tumefaciens* cells were induced for 14 h with 2 μM acetosyringone, resuspended in K3 medium, incubated for 2–3 h at 25°C, centrifuged, and then cocultivated with regenerating tobacco protoplasts (10^8 bacteria per ml; 10^5 protoplasts per ml) for various periods of time (24). Protoplasts (≈10^6) were pelleted by low-speed centrifugation, washed with K3 medium containing 0.4 M glucose, and resuspended in 1.5 ml of 10 mM Tris·HCl, pH 5.6/5 mM EDTA. After incubation at room temperature for 3 min, the protoplast lysate was centrifuged in a microcentrifuge for 5 min. The top 250 μl of solution was discarded, and the next 500 μl was incubated with 1.0% proteinase K (Sigma) and 0.5% SDS for 5 min at 37°C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation. Nucleic acids (mostly RNA; no DNA was detectable) were resuspended in 10 mM Tris·HCl, pH 7.5/1 mM EDTA. For PCR amplification of untreated and S1 nuclease-treated samples, equal amounts of solution were taken. For S1 nuclease digestion, samples were treated with 5 units of S1 nuclease (Amersham) for 10 min at 37°C. DNA was amplified in a reaction mixture (100 μl) containing 2 units of HotTub polymerase (Amersham), 5.0% dimethyl sulfoxide, 10 μmol of bovine serum albumin, 50 μmol of dNTPs (Amersham), and 20 pmol of primers. Amplification was performed for 40 cycles as follows: 4 min at 94°C, 1 min at 52°C, and 2 min at 72°C for the first cycle; 2 min at 94°C, 1 min at 52°C, and 2 min at 72°C for 38 cycles; 2 min at 94°C, 1 min at 52°C, and 10 min at 72°C for the final cycle. Primers for the *tms* genes were as follows: 5′-CGAGGTAATGGCCACCATCTCTCTG-3′ and 5′-GGTGCCAAGTCGATGGCAACGAGAC-3′ (26); for *picA*, 5′-ATGCGCATGAGGCTCGTCTTCGAG-3′ and 5′-GACGCAACGCATCCTCGATCAGCT-3′ (27); for *virA*, 5′-TCTACGGTCATGGTCCACTAGACG-3′ and 5′-TGCTGCTCAACTGCTACGCCAGCT-3′ (28). PCR products were analyzed by electrophoresis through 1.0% agarose gels.

RESULTS

Disappearance of T-Strands from *A. tumefaciens* Bound to Plant Cells. To investigate whether T-strands disappear from *A. tumefaciens* after binding to plant cells, we first induced *vir* gene activity and T-strand production in *A. tumefaciens* At493 with 100 μM acetosyringone for 14 h. β-Galactosidase activity assays revealed the induction of the *pinF*::*lacZ* fusion harbored by this strain, and DNA blot analysis (19) revealed the accumulation of a high level of T-strands (data not shown). Induced bacterial cells were incubated in K3 plant tissue culture medium lacking acetosyringone in the presence or absence of *N. tabacum* protoplasts. At various times DNA was isolated from the bacteria and subjected to blot analysis using a T-DNA fragment as a hybridization probe. Fig. 1 shows the results of such an analysis in which the DNA was transferred to the membrane under denaturing conditions. Using these conditions, both processed single-stranded T-DNA molecules (T-strands) and T-DNA within the Ti plasmid could be detected (19). Comparison among the lanes of the signal seen in the Ti-plasmid region of the blot indicated that equal amounts of DNA were transferred to the membrane (in Fig. 1, the Ti-plasmid region of the blot was cut off for clarity of presentation). Rehybridization of the blot with a probe homologous to the plant rRNA genes indicated that DNA isolated from bacteria bound to plant cells was not detectably contaminated by plant DNA (data not shown). There was a rapid and binding-dependent loss of T-strands from preinduced *A. tumefaciens* cells. When bacteria were incubated in K3 medium without tobacco protoplasts, T-strand levels were relatively constant for 2 h, after which their steady-state concentration declined (lanes 1, 4, 7, and 10). In bacteria incubated with but not tightly bound to protoplasts (i.e., bacteria separable from protoplasts by filtration), the steady-state level of T-strands was constant for 1 h and slightly decreased by 2 h, after which their steady-state concentration also declined (lanes 2, 5, 8, and 11). In contrast, the concentration of T-strands in bacteria that were tightly associated with protoplasts declined rapidly. Densitometric scanning of the autoradiogram indicated that within 30 min, the steady-state level of T-strands decreased to <25% that of unbound bacteria (lanes 3, 6, 9, and 12). Within 2 h, T-strand levels had further declined to ≈5% that of unbound bacteria. One possible explanation of these data is that within this short period of time T-strands transferred from the bacteria to bound plant cells. An alternative explanation for this result is that a binding-dependent single-stranded nuclease activity within the bacteria degraded T-strands. We do not favor this latter explanation for two reasons: (*i*) T-strands are probably coated with the VirE2 single-stranded DNA binding protein (12, 29, 30) that most likely protects the DNA from nuclease activity. If a nuclease were present then T-strands from a *virE* mutant that lacks VirE single-stranded DNA binding protein should not be as stable as T-strands from a *pinF* mutant that has T-strands protected by VirE protein. *A. tumefaciens virE* mutants accumulate wild-type levels of T-strands after induction with acetosyringone (31) or tobacco protoplasts (24), and these mutants show kinetics of T-strand loss similar to that of the *pinF* mutant cells (data not shown). Therefore, such a hypothetical nuclease activity is unlikely to exist. (*ii*) The kinetics of loss of T-strands from protoplast-bound bacterial cells parallels the kinetics of entry of T-strands into tobacco cells (see below).

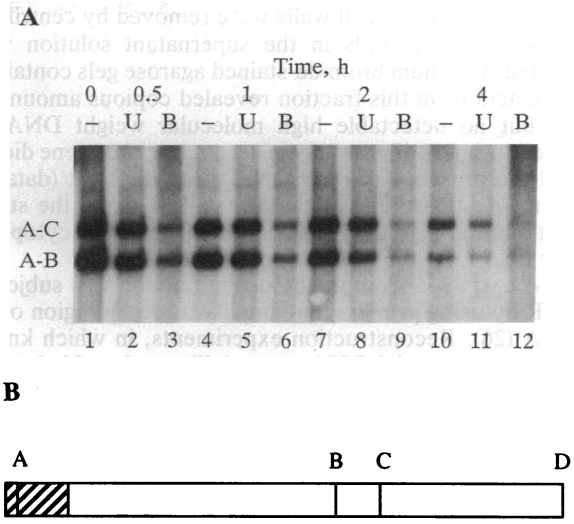

FIG. 1. DNA blot analysis of the disappearance of T-strands from *A. tumefaciens* preinduced with acetosyringone. (*A*) Analysis of *A. tumefaciens* DNA after transfer of the bacteria to K3 tissue culture medium. Lanes: 1, 4, 7, and 10, DNA from bacteria incubated in the absence of protoplasts (−); 2, 5, 8, and 11, bacteria incubated with but not bound to protoplasts (U); 3, 6, 9, and 12, bacteria bound to protoplasts (B). Time of incubation after withdrawal of acetosyringone is indicated above the lanes. A-C and A-B, positions of migration of the single-stranded T-DNA molecules extending from T-DNA borders A to B and A to C (19, 24). Only the T-strand region of the gel is shown. (*B*) T-DNA of pTiA6 harbored by *A. tumefaciens* At493. T-DNA borders are labeled A, B, C, and D. Hatched region shows hybridization probe used.

Association of T-Strands with Tobacco Protoplasts. To investigate whether T-strands that disappeared from bacteria reappeared in plant protoplasts, we cocultivated acetosyringone-induced *A. tumefaciens* At493 with tobacco protoplasts. After various periods of time, we separated unbound bacteria from the cocultivation mixture and lysed the protoplasts by osmotic shock. Plant cell organelles (nuclei, mitochondria, and chloroplasts) and bacterial cells remaining

attached to the plant cell walls were removed by centrifugation, and nucleic acids in the supernatant solution were extracted. Ethidium bromide-stained agarose gels containing nucleic acid from this fraction revealed copious amounts of RNA but no detectable high molecular weight DNA. In addition, primers directed against a nuclear *rbcS* gene did not reveal the presence of *rbcS* DNA using the PCR (data not shown). We therefore conclude that DNA from the supernatant fraction most likely came from the plant cell cytoplasm rather than from the nucleus or other organelles.

DNA extracted from the cytosolic fraction was subjected to PCR by using primers that amplify a 695-bp region of the T-DNA (26). Reconstruction experiments, in which known amounts of bacterial DNA were deliberately added to the PCR amplification mixture, indicated that our PCR protocols were sensitive enough to detect one to a few molecules per reaction (data not shown). A 695-bp fragment could be amplified from total DNA isolated from induced bacterial cells (Fig. 2*A*, lane 1). DNA isolated from plant cells cocultivated with *A. tumefaciens* At493 for 0.5, 2, and 5 h also yielded this 695-bp fragment (lanes 5–7). No 695-bp fragment could be amplified from the supernatant solution of bacterial cells (lane 2) or uninfected plant cells (lane 3) extracted in a similar manner or from plant cells mixed with induced bacterial cells and nucleic acids immediately extracted (0 time point; lane 4). These results suggest that T-DNA molecules become associated with the cytosolic fraction of plant cells within 30 min of cocultivation. In nine independent cocultivation experiments, we have consistently seen more PCR product by using DNA from a 2-h cocultivation than by using DNA from 0.5- or 5-h cocultivations. Even though our PCRs were not necessarily quantitative, this consistency in observing more PCR product from a 2-h cocultivation than from a 0.5-h cocultivation (using *A. tumefaciens* At493) suggests that T-DNA molecules continue to accumulate in the plant cytoplasm for >30 min. The decrease in T-DNA after 2 h could result either from degradation of T-DNA in the cytoplasm or from transport of T-DNA to the nucleus.

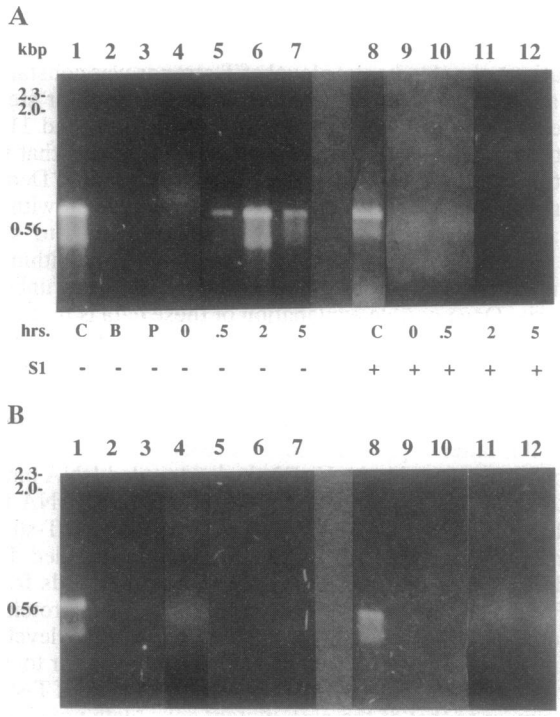

FIG. 2. PCR detection of single-stranded T-DNA molecules from *A. tumefaciens* At493 (*pinF*::*lacZ*) associated with tobacco protoplasts. PCR amplification was performed on the following samples: lane 1, 200 ng of total induced bacterial DNA (C); lane 2, DNA from the supernatant solution from osmotic shock-treated bacteria (B); lane 3, DNA from uninfected protoplasts (P); lanes 4–7, DNA from protoplasts infected for 0, 0.5, 2, and 5 h, respectively; lane 8, 50 ng of total induced bacterial DNA (C); lanes 9–12, DNA from protoplasts infected for 0, 0.5, 2, and 5 h, respectively. Lanes 8–12, DNA was treated with S1 nuclease prior to PCR amplification. (*A*) PCR primers were directed against the T-DNA *tms* loci. (*B*) PCR primers were directed against the *A. tumefaciens picA* chromosomal locus. Size markers are from phage λ DNA digested with *Hin*dIII.

To determine whether T-DNA associated with protoplasts was single or double stranded, we treated *A. tumefaciens* DNA, or DNA from the supernatants of the 2- and 5-h cocultivations, with S1 nuclease prior to PCR amplification. Although the 695-bp fragment was detected by using total induced *A. tumefaciens* DNA digested with S1 nuclease (Fig. 2, lanes 8), no fragment was amplified by using the S1 nuclease-treated cocultivation samples (lanes 9–12). In the control experiment shown in lane 8 (and in the control, but not experimental, samples described below), ≈4-fold more DNA was used as an initial PCR template for the untreated DNA than for the S1 nuclease-treated DNA. Our results suggest that, under the conditions described in *Materials and Methods*, there was no detectable double-stranded DNase activity in our S1 nuclease preparation. This conclusion was confirmed by treatment of *in vitro* labeled double-stranded DNA with S1 nuclease. Using a 10-fold greater concentration of S1 nuclease than that used for our experimental samples, we could not detect any degradation of labeled double-stranded DNA (data not shown). Taken together, these data show that we could detect the association of only single-stranded T-DNA with protoplasts.

To confirm that T-DNA detected in the PCR assays originated from infected plant cells and not from lysed bacteria, we performed PCR analyses on the supernatant fractions of the cocultivation samples using primers directed against the Ti plasmid-encoded *virA* gene (28) and the chromosomal *picA* gene (27). Neither of these sets of primers revealed the presence of bacterial Ti plasmid or chromosomal DNA in our samples (Fig. 2*B*; data not shown). Reconstruction reactions indicated that these primers function at a level of sensitivity similar to that of the T-DNA primers (data not shown). These controls confirm that the T-DNA detected in the cocultivation samples did not originate from lysed bacteria.

Analysis of the Association of T-Strands from *A. tumefaciens virE* Mutants with Protoplasts. The *virE2* gene encodes a single-stranded DNA binding protein that is thought to coat T-strands and protect them from endonucleases (11, 12, 29, 30). We performed a PCR assay using DNA isolated from cytosolic fractions of protoplasts cocultivated with the *A. tumefaciens virE* mutant At495 (Fig. 3). This strain accumulates near-wild-type levels of T-strands after induction by tobacco protoplasts or acetosyringone but is severely attenuated in virulence (23, 24, 31). The 695-bp T-DNA fragment was amplified from the DNA of both the positive control (Fig. 3*A*, lane 1) as well as the cocultivated samples (lanes 5 and 6) when the *tms* primers were used. Controls in which the DNA was digested with S1 nuclease prior to amplification (lanes 7–9) or using primers directed against the *picA* gene (Fig. 3*B*) indicated that the amplified DNA was single stranded and did not derive from lysed bacteria. The quantity of PCR product from the cocultivation experiment using the *A. tumefaciens virE* mutant was consistently less than that from cocultivations using the *pinF* mutant (in Fig. 3, a 5-fold greater volume from the amplification reaction was loaded onto the gel than was loaded from the amplification reaction using DNA from protoplasts cocultivated with the *A. tumefaciens pinF* mutant in Fig. 2). Our results suggest that either fewer T-strands associate with protoplasts following cocultivation with *A. tumefaciens virE* mutant cells or that T-strands from this strain were more susceptible to nucleolytic degradation in the plant cytoplasm.

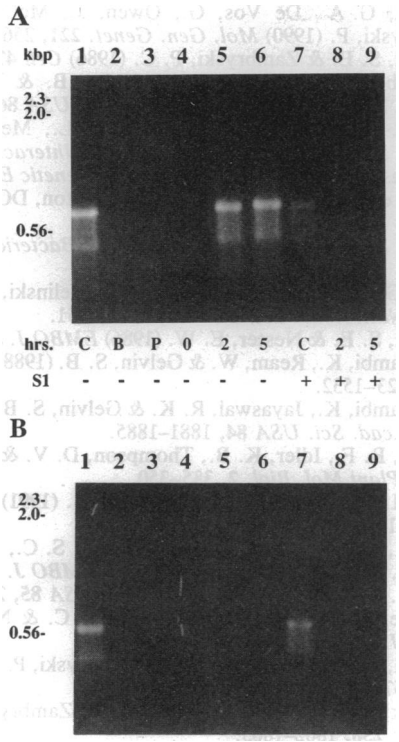

FIG. 3. PCR detection of single-stranded T-DNA molecules from *A. tumefaciens* At495 (*virE*::*lacZ*) associated with tobacco protoplasts. PCR amplification was performed on the following samples: lane 1, 200 ng of total induced bacterial DNA (C); lane 2, DNA from the supernatant solution from osmotic shock-treated bacteria (B); lane 3, DNA from uninfected protoplasts (P); lanes 4–6, DNA from protoplasts infected for 0, 2, and 5 h, respectively; lane 7, 50 ng of total induced bacterial DNA (C); lanes 8 and 9, DNA from protoplasts infected for 2 and 5 h, respectively. Lanes 7–9, DNA was treated with S1 nuclease prior to PCR amplification. (*A*) PCR primers were directed against the T-DNA *tms* loci. (*B*) PCR primers were directed against the *A. tumefaciens picA* chromosomal locus. Size markers are from phage λ DNA digested with *Hin*dIII.

Analysis of the Association of T-Strands from *A. tumefaciens virB* Mutants with Protoplasts. The *virB* operon of *A. tumefaciens* encodes 11 proteins, most of which are localized to the bacterial membrane (14–16). These proteins are thought to make up the channel through which T-DNA is transferred to plant cells. *A. tumefaciens virB* mutants are avirulent, yet they accumulate wild-type levels of T-strands (23, 24, 31). We attempted to detect the association of T-DNA from an *A. tumefaciens virB* mutant with tobacco protoplasts. Fig. 4 shows that, although we could detect T-DNA from the *A. tumefaciens pinF* mutant At493 associated with protoplasts (lanes 2 and 3), in a parallel experiment we could not detect such T-DNA association following cocultivation of protoplasts with the *A. tumefaciens virB* mutant At494 (lanes 5 and 6). The 695-bp T-DNA fragment could be amplified from total DNA of *A. tumefaciens* At494 (lane 4). The T-DNA detected in the cytoplasm of protoplasts cocultivated with *A. tumefaciens pinF* mutant cells did not result from lysed bacteria because we could not detect DNA from the *virA* locus in these samples (lanes 10 and 11). This experiment indicates that VirB proteins are involved in the association of T-DNA with plant cells.

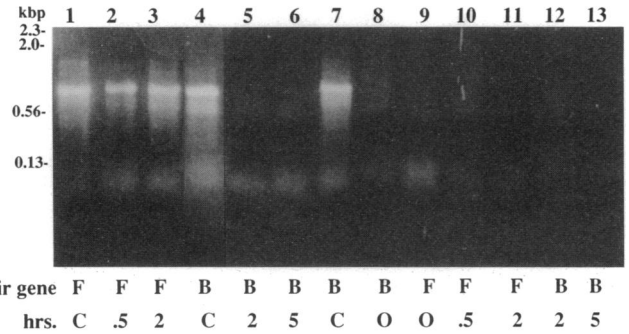

FIG. 4. PCR detection of single-stranded T-DNA molecules from *A. tumefaciens* At494 (*virB*::*lacZ*) and *A. tumefaciens* At493 (*pinF*::*lacZ*) associated with tobacco protoplasts. PCR amplification was performed on the following samples: 100 ng of total bacterial DNA (C) (lanes 1, 4, and 7); DNA from protoplasts infected for 0.5 h (lanes 2 and 10), 2.0 h (lanes 3, 5, 11, and 12), and 5.0 h (lanes 6 and 13); supernatant solution from osmotically shocked bacteria (O) (lanes 8 and 9). Lanes 1–6, PCR primers were directed against the *tms* genes; lanes 7–13, PCR primers were directed against the Ti plasmid-localized *virA* gene; lanes 1–3 and 9–11, *A. tumefaciens* At493 was used; lanes 4–8, 12, and 13, *A. tumefaciens* At494 was used. Size markers are from phage λ DNA digested with *Hin*dIII.

DISCUSSION

The transfer of T-DNA from *A. tumefaciens* to plant cells is a unique example of the natural exchange of genetic material from a prokaryote to a eukaryote. Previous studies have shown that, following induction with acetosyringone, both single- and double-stranded processed T-DNA molecules accumulate in *A. tumefaciens* (7, 18, 19). Although current models favor the transfer of single-stranded T-DNA molecules (T-strands), perhaps as a DNA–protein complex (T-complex; ref. 13), no study has yet shown which processed T-DNA molecule (single or double stranded) is the transferred form. Our data suggest that T-DNA is transferred to plant cells as a single-stranded DNA molecule and strengthen the model that the transfer of T-DNA between bacterial and plant cells resembles the conjugal transfer of DNA between bacterial cells (17). Because our DNA isolation method utilized protease digestion and phenol extraction, we are unable to conclude that T-strands enter plant cells as a nucleoprotein complex. T-strands accumulate in *virE* mutant *A. tumefaciens* cells to approximately the same extent as they do in wild-type and *pinF* mutant bacterial cells (24, 31), yet they accumulate in the cytoplasm of infected protoplasts to a considerably lower extent than do T-strands from *pinF* mutant bacteria. These results suggest that VirE2 protein may protect T-strands from nucleolytic degradation in the plant. We speculate that VirE2 protein is important for T-DNA transport to the nucleus (32) and/or VirE2 protein is necessary to protect T-strands from nuclease degradation in the plant cytoplasm.

Our conclusions regarding T-strand association with plant cells are predicated upon the isolation of T-DNA molecules from the plant cytoplasm in the absence of bacterial cell lysis. We have found that osmotic lysis of regenerating protoplasts does not permit the detectable leakage of DNA from contaminating bacterial cells. This latter conclusion is based on our inability to detect genes encoded by the bacterial chromosome (*picA*) or the Ti plasmid (*virA*) with this lysis treatment. The validity of our experiments is strengthened by our lack of ability to detect T-strands from an *A. tumefaciens virB* mutant associated with cocultivated protoplasts. Many of the VirB proteins have been localized to the bacterial membrane, and some have homology to proteins that, in other bacteria, aid in the export of proteins from the bacterial cell (33). *virB* mutant *A. tumefaciens* strains are avirulent (23), and VirB proteins may participate in the export of T-strands to the plant. Our results are consistent with this hypothesis.

V.M.Y., T.R.S., and V.G. contributed equally to this work. The

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