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Agrobacterium VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells

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ABSTRACT ***Agrobacterium*** **genetically transforms plant cells by transferring a single-stranded DNA (ssDNA) copy of the transferred DNA (T-DNA) element, the T-strand, in a complex with *Agrobacterium* proteins VirD2, bound to the 5′ end, and VirE2. VirE2 binds single-stranded nucleic acid cooperatively, fully coating the T-strand, and the protein localizes to the plant cell nucleus when transiently expressed. The coupling of ssDNA binding and nuclear localizing activities suggests that VirE2 alone could mediate nuclear localization of ssDNA. In this study, fluorescently labeled ssDNA accumulated in the plant cell nucleus specifically when microinjected as a complex with VirE2. Microinjected ssDNA alone remained cytoplasmic. Import of VirE2–ssDNA complex into the nucleus via a protein import pathway was supported by (*i*) the inhibition of VirE2–ssDNA complex import in the presence of wheat germ agglutinin or a nonhydrolyzable GTP analog, both known inhibitors of protein nuclear import, and (*ii*) the retardation of import when complexes were prepared from a VirE2 mutant impaired in ssDNA binding and nuclear import.**

Agrobacterium tumefaciens is the most widely utilized vector for the genetic transformation of plants. The study of the mechanisms underlying this complex process has revealed fundamental concepts in several areas, from plant–bacteria signaling to conjugation to nuclear import. In the final phases of transformation, the transfer intermediate, thought to be a protein–single-stranded DNA (ssDNA) complex, is transported into the plant cell nucleus. Here we provide evidence that the protein component of the complex mediates nuclear import of the ssDNA. The study of this protein-mediated nuclear uptake of ssDNA may provide insight into other processes where single stranded nucleic acid passes through the nuclear pore and may aid in the design of improved methods for transformation of eukaryotic cells.

In nature, *A. tumefaciens* is the agent of crown-gall, a disease of dicotyledonous plants in which a specific segment of bacterial DNA [transferred DNA (T-DNA)] is transferred and integrated into the plant genome and its encoded products elicit the tumorous phenotype (reviewed in refs. 1 and 2). The transferred element resides on the tumor-inducing (Ti) plasmid and is defined and delimited by 25-bp direct repeats at its ends. In the presence of specific small phenolic molecules released by wounded plants, *Agrobacterium* initiates gene expression from the Ti-plasmid virulence (*vir*) region, which produces most of the trans-acting factors for DNA transfer. Products of *vir* genes act at the T-DNA borders to generate a linear single-stranded copy of the T-DNA (T-strand) and mediate its transfer from the bacterium to the plant cell. The single-stranded nature of the transfer intermediate was first proposed based on studies in *Agrobacterium* (3). Recent studies of the T-DNA on its arrival in the plant cell also strongly support the hypothesis that the transfer intermediate is single-stranded (4, 5).

Further, during its transit, the T-strand is not free ssDNA, but evidence to date suggests that it is in a complex (T-complex) with two Vir proteins, VirD2 and VirE2 (reviewed in ref. 6). A single molecule of the VirD2 endonuclease is tightly associated with the 5′ end of the T-strand. VirE2, a ssDNA-binding protein that binds tightly and cooperatively without sequence specificity, coats the length of the T-strand (7–10). During infection, the T-complex is mobilized out of the bacterium and into the plant cell, where the T-strand is eventually integrated into the genome of the infected cell.

Prior to integration, the T-complex enters the plant cell nucleus by a mechanism not yet fully characterized (reviewed in refs. 1 and 2). The estimated size of the T-complex exceeds the size-exclusion limit, ≈40 kDa, of the nuclear pore and thus precludes the possibility of it entering the nucleus by passive diffusion. For instance, the 20-kb T-strand produced from the nopaline Ti-plasmid would bind ≈600 molecules of VirE2 (60.5 kDa) and one VirD2 (47 kDa), yielding a complex with a total mass of 50×10^6 Da (2). To cross the nuclear membrane, this large complex most likely exploits endogenous cellular machinery for nuclear import, which would necessitate nuclear localization signals (NLSs). Any of the three components of the T-complex (T-strand, VirD2, and VirE2) could potentially provide the signal(s) that identifies the T-complex as a substrate for nuclear import. However, T-strand is the least likely to contribute an NLS, since any DNA between the T-DNA borders can serve as the substrate for T-strand production. Therefore, nuclear targeting signals were predicted to reside in the proteins of the complex, and T-strand would then be cotransported into the nucleus by virtue of these associated proteins.

In support of this hypothesis, both VirD2 and VirE2 localize to the nucleus when transiently expressed in tobacco protoplasts. VirD2 has a bipartite NLS in the C-terminal 37 amino acids (11). The positioning of the single VirD2 molecule at the 5′ end of the T-strand suggests that polar uptake of the T-complex into the nucleus may be a necessary prerequisite to integration or possibly a standard characteristic of nucleoprotein complex transport through the nuclear pore. However, deletion of the VirD2 NLS reduces but does not completely abolish tumorigenicity (12), indicating that some of the functions of VirD2 in the transfer of the T-complex (e.g., targeting to the nucleus) may be partially fulfilled by VirE2. Indeed, VirE2 contains two bipartite NLSs, both of which are required for maximally efficient nuclear import of VirE2 in tobacco protoplasts (13). An *in planta* role for VirE2 is further supported by transgenic plant studies (14) where a nontumori-

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Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; GMP-*P*[NH]*P*, 5′-guanylyl imidodiphosphate; NLS, nuclear localization signal; T-complex, transferred complex: T-DNA, transferred DNA; Ti-plasmid, tumor-inducing plasmid; WGA, wheat germ agglutinin.

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genic *virE2*$^-$ *Agrobacterium* was restored to virulence when inoculated on a transgenic tobacco plant expressing wild type VirE2 (13).

In the nuclear targeting studies described above, transiently expressed VirD2 and VirE2 were presumably monomeric and not complexed with single-stranded nucleic acid. Thus, the ability of these proteins to target ssDNA to the nucleus remained unconfirmed. To test the hypothesis that VirE2 mediates the nuclear localization of single-stranded nucleic acid by coupling the functions of nuclear localization and ssDNA binding, proteins–ssDNA complexes were generated *in vitro* from purified VirE2 and fluorescent ssDNA. *In vitro* formed nucleoprotein complexes were microinjected into stamen hair cells of the flowering plant *Tradescantia virginiana*, and their intracellular movement was followed by epifluorescence microscopy.

MATERIALS AND METHODS

Preparation of Fluorescent DNA. To generate fluorescent DNA, 112 ng of an 800-bp restriction fragment from the coding sequence for tobacco mosaic virus movement protein (V.C., unpublished data) was used as the template in a PCR reaction (final volume = 100 μl). 5′ and 3′ 28-base primers were used at a final concentration of 0.5 μM in 1× *Taq* polymerase incubation buffer (Boehringer Mannheim) supplemented with 5 mM $MgCl_2$; dATP, dCTP, and dGTP were used at 100 μM each. A mixture of dTTP and fluorescein-12-dUTP was used at a combined concentration of 100 μM. The molecular ratio of dTTP to fluorescein-12-dUTP was either 7:3 (Boehringer Mannheim fluorescein-12-dUTP) or 99:1 (Perkin–Elmer fluorescein-12-dUTP). *Taq* polymerase (Boehringer Mannheim) was used at 0.5 unit per system. The thermal cycle program was 1 cycle of 95°C for 2 min, 55°C for 1.5 min, and 72°C for 1.5 min; 35 cycles of 95°C for 1 min, 55°C for 1.5 min, and 72°C for variable periods of time as follows: 2 min (10 cycles), 2.5 min (10 cycles), 3 min (10 cycles), and 3.5 min (5 cycles); and 1 cycle of 72°C for 7 min. The product was purified on a Nuc-Trap column (Stratagene) according to the manufacturer's instructions, precipitated with ethanol, and resuspended in 10 μl of 10 mM Hepes buffer (pH 7.0). The final concentration of the product was 50–100 ng·μl^{-1}.

VirE2 Expression, Purification, and Binding Activity. VirE2 overproduction and purification and ssDNA binding activity assays were performed as described (13). VirE2 binds cooperatively to both fluoresceinated ssDNA and unlabeled ssDNA (Fig. 1).

Microinjection. Microinjection needles were pulled from filamented microcapillary tubes (World Precision, Inc., 1.0 mm × 0.58 mm) on a micropipette puller (Sutter Instruments, model P-87). The needles were pulled to a tip outer diameter of ≈1 μm measured by the method of Mittman *et al.* (15). Solutions for microinjection were loaded into the needle by backfilling. To minimize clogging of the injection needle, a tip outer diameter of 1 μm was required and the protein/ssDNA (wt/wt) ratio could not exceed 10. At weight ratios >10, complexes formed aggregates too large to pass through the needle tip. Under the conditions used, VirE2–ssDNA binding was not saturated. Since VirE2 binds cooperatively, most of the ssDNA was completely bound by VirE2 at the protein/ssDNA ratio employed in this study. However, a small fraction of the DNA was almost completely uncoated by VirE2, resulting in some residual cytoplasmic fluorescence in all experiments. The needle was positioned for microinjection by Narishige coarse (model MN-3) and fine (models MW-3 and MW-4) micromanipulators.

For microinjection, individual stamen were excised from immature flower buds of *Tradescantia virginiana* and immobilized in a thick film of low-gelling-temperature agarose (3%, wt/vol) with 3% (wt/vol) sucrose, 5 mM KCl, 0.1 mM $CaCl_2$, and 5 mM Hepes (pH 7.0) (16). The stamen hairs were teased apart, and cells toward the base of the stamen hairs were selected for microinjection since they were no longer undergoing cell division and nearly expanded to their full size (Fig. 2*A*). After the cell was impaled, solutions were introduced into the cell by pressure injection (World Precision Instruments, pneumatic PicoPump, model PV820; Sarasota, FL) for 5 min. Approximately 1 pl is estimated to be microinjected. Thus, 0.05–0.1 pg of fluorescent DNA is introduced into the cell. Withdrawal of the needle required at least 15 min to allow the cell to seal the puncture (16). During this time, the system could not be disturbed. Consequently, the earliest time at which an image could be captured was 15 min after the pressure injection was stopped. Microinjections and subsequent observations were performed on a Zeiss Axiophot epifluorescence microscope. Images were captured with a cooled charge-coupled device (CCD; Princeton Instruments model TEA/CCD1400-TK; Trenton, NJ) controlled by IPLab (Signal Analytics, Vienna, VA). Digital images were processed and figures were assembled on a Power Macintosh 8100/80 using Adobe Photoshop (Adobe Systems, Mountain View, CA).

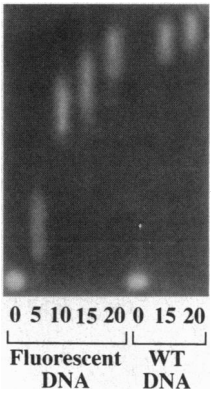

FIG. 1. ssDNA binding of VirE2 to DNA containing fluorescein-12-dUTP and to wild-type (WT) DNA. VirE2–ssDNA complexes are detected by ethidium bromide fluorescence. Prior to addition of purified VirE2, 125 ng dsDNA per reaction was denatured by boiling for 3 min and chilling on ice for 5 min. The number under each lane indicates the weight ratio of VirE2 to DNA. VirE2 and denatured DNA were incubated for 10 min at 4°C (8). Incubation reactions were separated on a 1% agarose TBE gel.

RESULTS

The pattern of fluoresence accumulation in a cell microinjected with DNA alone is dramatically different from that of a cell microinjected with VirE2 nucleoprotein complex. When the focal plane is at the equator of the nucleus in a cell microinjected with DNA alone, the nucleus appears as a dark circle highlighted by a fluorescent ring (Fig. 2*B*). The ring results from a peripheral layer of cytoplasm around the nucleus that contains fluorescently labeled ssDNA. The dark appearance of the nucleus in this cell indicates that any fluorescence in the cytoplasm above or below the plane of focus does not interfere with unequivocal detection of cytoplasmic or nuclear localized fluorescence. Therefore, denatured fluoresceinated DNA alone does not appreciably accumulate in the nucleus (Fig. 2*B*). Even after 90 min, the nucleus is still identifiable by its darker appearance (Fig. 4*A*). After 3 h fluorescence was still cytoplasmic and no appreciable accumulation was observed in the nucleus, suggesting that the DNA was undegraded (data not shown).

In a cell injected with the VirE2–ssDNA complex, the nucleus appears as a solid fluorescent circle, indicating that the fluorescent DNA has accumulated inside the nucleus (Fig.

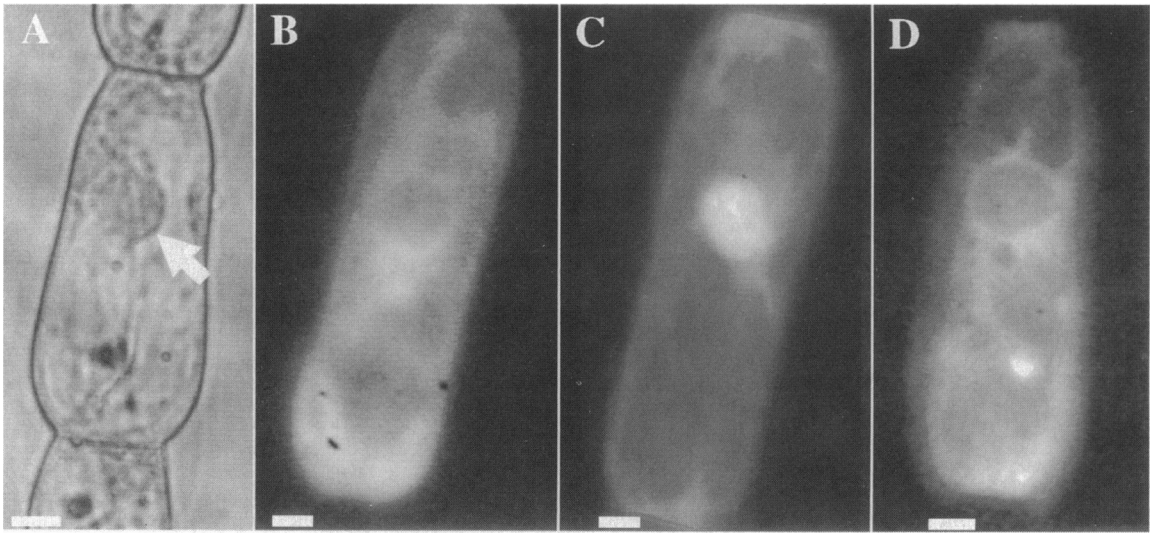

FIG. 2. Localization of microinjected fluorescent DNA in stamen hair cells of *Tradescantia virginiana*. (*A*) Differential interference contrast photomicrograph of stamen hair cell. The arrow indicates the nucleus. (*B*) ssDNA alone. (*C*) VirE2–ssDNA complex. (*D*) dsDNA and VirE2 comicroinjected. (Bars = 10 μm.)

2*C*). Given that ssDNA complexed with VirE2 is highly resistant to degradation by nucleases (17), it is unlikely that fluoresence in the nucleus results from degradation of complexed DNA. When fluorescent DNA is introduced in a complex with VirE2, nuclear import of fluorescence is detected within 15 min of microinjection (Fig. 2*C*). Fluoresceinated double-stranded DNA (dsDNA), to which VirE2 does not bind, does not accumulate in the nucleus when coinjected with VirE2 (Fig. 2*D*). This suggests that VirE2 cannot facilitate nuclear import of nucleic acid in the absence of binding, for example by opening the nuclear pore and allowing diffusion of the DNA. Similar results were consistently obtained in replicate experiments (Table 1). Therefore, VirE2 specifically mediates nuclear accumulation of ssDNA, and this is dependent on the formation of a nucleoprotein complex.

In general, little is known about the import of nucleoprotein complexes into the nucleus. An exception is the nuclear import of small nuclear ribonucleoprotein particles (snRNPs) where multiple kinetic pathways for nuclear import have been described (18–20) that utilize either amino acid or nucleotide sequences as NLSs. Because previous work suggested that nuclear targeting information resides in the protein components of the T-complex, nuclear import of the VirE2–ssDNA complex was characterized in the presence of wheat germ agglutinin (WGA) or 5′-guanylyl imidodiphosphate (GMP-*P*[NH]*P*), a nonhydrolyzable GTP analog. Both of these compounds are known to inhibit specifically the nuclear import of protein in animal systems.

In animal-based nuclear import systems, WGA blocks nuclear import of karyophilic proteins by binding to glycoproteins that constitute part of the nuclear pore; it appears to permit nuclear-targeted proteins to bind to the nuclear pore but prevents the translocation of these proteins across the nuclear membrane into the nucleus (21). Microinjected fluorescein-labeled WGA also binds to plant cell nuclei (Fig. 3 *A* and *B*), which suggests that it could be used to inhibit nuclear import in plant systems. Because WGA is smaller than the size exclusion limit of the nuclear pore, the excess can diffuse into the nucleus after all of the nuclear pore binding sites are occupied. A confocal image confirmed binding of the WGA at the periphery of the nucleus, most likely on the nuclear membrane (data not shown). In the presence of unlabeled WGA, the VirE2–ssDNA complex does not accumulate inside the nucleus within the first 15 min (Fig. 3*C*) compared with non-WGA-treated controls (Fig. 2*C*). This inhibition of import is detected only when WGA is injected prior to injection of complex (needle concentration = 20 mg·ml^{-1}). Since two microinjections are required for this experiment, ≈20 min elapse between microinjection of the WGA and introduction of the complex. WGA does not effectively block nuclear import when coinjected with complex. Presumably, this time differential is required for WGA to accumulate at the nuclear envelope to a level sufficient to inhibit import. Inhibition of nuclear import continues up to 2 hr, after which some nuclear accumulation of fluorescent complex could occasionally be detected (data not shown). Possibly, import resumed following WGA turnover. Alternatively, WGA may not completely block nuclear import but only reduce nuclear import rate so that >2 hr are required for detectable levels of fluorescence to accumulate in the nucleus.

Table 1. Summary of microinjection results

Injection substrate	Complex formation	Cell ratio*	Fig. 2 panel
Denatured DNA	No	0/5	*B*
+ VirE2	Yes	7/7	*C*
Nondenatured DNA + VirE2	No	0/3	*D*

*Number of cells with nuclear localized fluorescence/total number of cells microinjected.

Nuclear import of the VirE2–ssDNA complex was also characterized in the presense of the nuclear import inhibitor GMP-*P*[NH]*P*. GMP-*P*[NH]*P* is a nonhydrolyzable GTP analog that interferes with Ran/TC4, the nuclear GTPase required for nuclear import of proteins in isolated nuclei and permeabilized cell systems. Ran/TC4 binds nonhydrolyzable GTP analogs irreversibly, which terminates the GTP cycle and possibly prevents Ran/TC4 from promoting either assembly and docking of a nuclear import complex or translocation through the nuclear pore (22, 23).

In contrast to WGA, GMP-*P*[NH]*P* inhibits nuclear import of the VirE2–ssDNA complex when coinjected with the complex (Fig. 3*D*). Based on a GMP-*P*[NH]*P* concentration of 50 mM in the needle and a presumed 100-fold dilution on microinjection (24), the intracellular concentration of GMP-*P*[NH]*P* is ≈0.5 mM. In permeabilized cell systems, GMP-*P*[NH]*P* concentrations of 0.1–1 mM are effective in blocking nuclear import of a protein substrate (23). As with WGA, nuclear import resumed after 2–3 hr (data not shown). Pos-

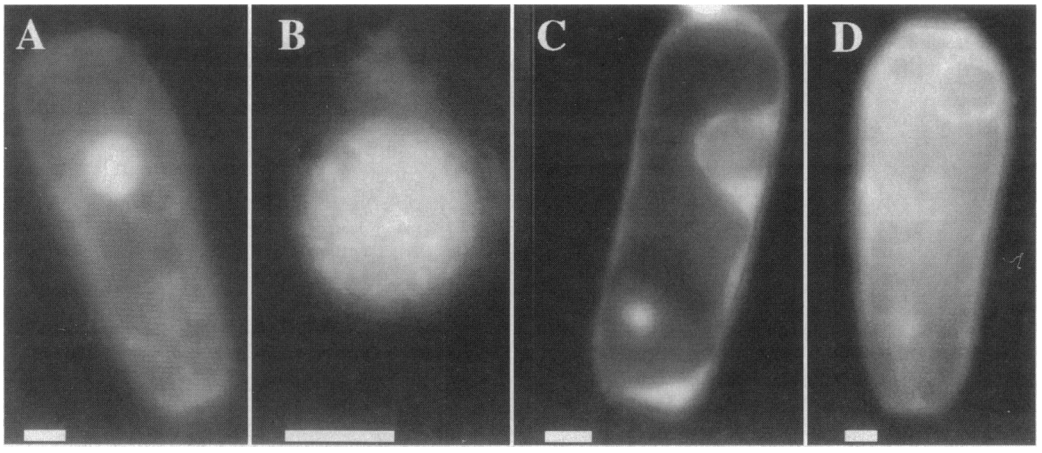

FIG. 3. Effect of nuclear import inhibitors on nuclear import of VirE2–fluorescein ssDNA complexes. (*A*) Nuclear accumulation of fluorescein-labeled WGA on and in nucleus. (*B*) Enlarged nucleus from *A*. (*C*) WGA (unlabeled) microinjected first followed by microinjection of VirE2–ssDNA complex. (*D*) Comicroinjection of VirE2–ssDNA complex and GMP-*P*[NH]*P*. (Bars = 10 μm.)

sibly, GMP-*P*[NH]*P* was degraded. Alternatively, since stamen hair cells are in cytoplasmic continuity via plasmodesmata, GMP-*P*[NH]*P* may be diluted by transport to adjacent cells. The size exclusion limits of plasmodesmata vary with cell type but have not been found to exceed 7 kDa—too small to allow diffusion of WGA (dimer is *ca.* 36 kDa) out of the injected cell.

Ideally, a more complete characterization of complex import would be facilitated by a mutant of VirE2 that binds single-stranded nucleic acid cooperatively but does not localize to the nucleus. However, Nuclear localizing and ssDNA binding activities are not completely separable. Substitution in or deletion of the C-terminal NLS reduces nuclear localization and abolishes ssDNA binding (13, 14). Substitution in or deletion of the N-terminal NLS also reduces nuclear localization (del C in ref. 13), but partial ssDNA binding activity is retained (13, 14). Dél C binds ssDNA, but not cooperatively, and is found in both the nucleus and cytoplasm. Nuclear import of VirE2–delC–ssDNA complex is considerably slower than nuclear import of complex formed with wild type VirE2 (Fig. 4 *B* and *C*). Significant nuclear import is evident within 15 min after microinjection of the wild-type VirE2 complex, and little change in nuclear accumulation of fluorescence is observed after 1 hr (Fig. 4*B*). In contrast, VirE2–delC complexes could not be detected in the nucleus until 90 min after microinjection (Fig. 4*C*). Nuclear accumulation of free DNA is not detected up to 90 min after microinjection (Fig. 4*A*). The lower rate of nuclear import observed for the VirE2–delC complex relative to the wild-type VirE2 complex correlates with the weaker NLS and reduced binding activity of the mutant. These data support the hypothesis that nuclear import of the T-complex occurs via a protein import pathway and requires tight association of VirE2 with the ssDNA.

DISCUSSION

The genetic transformation of plant cells by *Agrobacterium* is dependent on the T-complex targeting to and through the bacterial cell inner/outer membranes and wall, the plant cell wall and plasma membrane, and the plant cell nuclear membrane. The presence of bipartite NLSs on VirD2 and VirE2, the protein components of the T-complex, suggests that the T-complex enters the nucleus via a protein import pathway. The present demonstration of VirE2-dependent nuclear accumulation of fluorescently labeled ssDNA strongly supports the hypothesis that VirE2 has a role in nuclear import of T-strand. We propose that, after entry into the plant cell, the proteins of the T-complex, VirD2 and VirE2, are recognized in the cytoplasm by NLS-binding proteins and targeted to the nucleus. Because of the tight association between these proteins and the T-strand, there is concomitant nuclear import of the T-strand.

The high molecular ratio of VirE2 to VirD2 in a wild-type T-complex raises the question of the necessity of VirD2 in the nuclear import of T-complex. In the case of the nopaline Ti-plasmid, the T-complex is predicted to have a VirE2/VirD2 molecular ratio of 600 (1, 2) and a VirE2 NLS/VirD2 NLS ratio of 1200. Deletion of the basic amino acids in the VirD2 NLS results in a 40% decrease in tumorigenicity, suggesting that VirD2 alone may not be sufficient for nuclear import of T-strand (12). A larger deletion in VirD2 that included the entire bipartite NLS and additional amino acids towards the C terminus resulted in almost complete loss of tumorigenicity (12). This might be explained by the recent finding that VirD2 is responsible for the precise integration of the T-DNA into a plant chromosome (25). Based on transient expression studies, Citovsky *et al.* (13) suggested that the NLS of VirD2 is intrinsically stronger than either NLS of VirE2 because two bipartite NLSs are required for complete nuclear localization of VirE2, whereas a single bipartite NLS is sufficient for complete nuclear localization of VirD2. The stronger VirD2 NLS is proposed to promote targeting of the T-complex to the nucleus in a polar fashion that insures translocation of the T-complex across the nuclear pore in a 5′ to 3′ direction (2). Polar translocation may be a common characteristic of transport through nuclear pores for naturally occurring nucleoprotein complexes (26). For example, nuclear export of a 75S premessenger ribonucleoprotein particle in *Chironomus tentans* also appears to initiate exclusively at the 5′ end of the RNA (27).

Thus, existing data are consistent with the requirement for both VirD2 and VirE2 in nuclear uptake of the T-complex (2). This role is not inconsistent with the additional functions of these proteins, nicking of the T-DNA borders on the Ti plasmid in *Agrobacterium* and integration into plant DNA in the case of VirD2, and cooperative binding to and protection of the T-strand in the case of VirE2. In the present context, it is interesting to speculate on additional roles of VirE2 that remain to be uncovered (for example, roles subsequent to nuclear import). Potential roles can be predicted from recent work indicating that the *vir* system is evolutionarily related to conjugation plasmids in the IncP group, especially RP4 (reviewed in ref. 28). In RP4, a broad host-range plasmid originally isolated from a strain of *Pseudomonas*, TraC1 is the ssDNA-binding protein and, thus, may perform a role comparable to that of VirE2. Once the DNA is transferred from donor to recipient, TraC1 acts as a primase to initiate con-

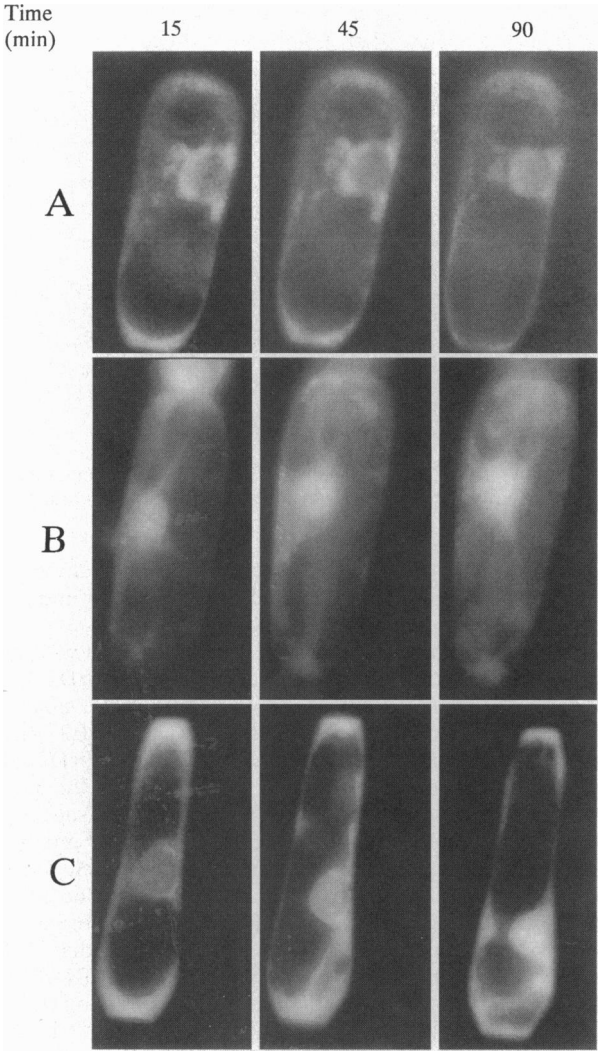

FIG. 4. Effect of mutations in VirE2 on nuclear import of VirE2–fluorescein ssDNA complex. The times indicated are the elapsed time after pressure injection was stopped. The earliest time at which an image could be captured was 15 min after cessation of pressure injection (see ref. 9). (*A*) ssDNA alone. (*B*) VirE2–ssDNA complex. (*C*) VirE2–delC–ssDNA complex. (Bar = 10 μm.)

version of single-stranded transferred DNA to dsDNA. Although primase activity has not been demonstrated for VirE2 *in vitro*, VirE2 is functionally analogous to TraC1 in other respects (29): both bind ssDNA and are transferred together with ssDNA to the recipient cell. By analogy, then, during T-strand integration into a chromosome, VirE2 may promote second-strand synthesis. Alternatively, VirE2 may employ a RecA-like mechanism, in which binding of ssDNA by VirE2 activates dsDNA binding and subsequent scanning for double-stranded regions homologous to its bound ssDNA (30). Short stretches of homology between the integration site and the ends of the T-DNA are consistent with this hypothesis (reviewed in ref. 2).

The present studies utilize predominantly biochemical and cell biological approaches to dissect one of the many steps involved in the genetic transformation of plant cells by *Agrobacterium*. In contrast, most previous studies utilized whole bacteria and assayed tumor formation in plants. In this context, the present data need to address earlier reports that (*i*) avirulent *virE2*$^-$, T-DNA$^+$ *Agrobacterium* can be complemented in the absence of conjugation by coinoculation with avirulent *virE2*$^+$, T-DNA$^-$ *Agrobacterium* (31, 32), leading to the suggestion that both VirE2 and the T-strand can enter the plant independently (32); and (*ii*) *virE2* mutants of *Agrobacterium* also are restored to virulence when inoculated on transgenic tobacco expressing wild-type VirE2 (13). In fact, the bacterial and *in planta* complementation results, as well as the present demonstration that VirE2 functions to direct ssDNA uptake from the cytoplasm to the nucleus, are all consistent with a major role of VirE2 in binding T-strand in the plant cytoplasm. Further, Yusibov *et al.* (5) found that fewer T-strands accumulated in the cytoplasm of plant cells infected with a *virE2* mutant strain of *Agrobacterium* than with a wild-type strain. Thus, they proposed that VirE2 functions in the cytoplasm to render T-strand resistant to exonucleolytic degradation (5), a function previously demonstrated *in vitro* that depends on the formation of a VirE2–ssDNA complex (17). Thus, the present results, while isolating only one step of T-strand transfer, are nevertheless consistent with existing data that suggest that VirE2 forms a complex with the T-strand prior to nuclear import.

The study of the mechanisms whereby *Agrobacterium* genetically transforms plant cells has provided insight into fundamental biological processes. In the present study, we have presented evidence that the Agrobacterium protein VirE2 can mediate the nuclear uptake of ssDNA in plant cells. As discussed above, this suggests that the transforming DNA from *Agrobacterium*, T-strand, is transported into the host plant cell nucleus by its associated proteins via endogenous protein nuclear import machinery. Similarly, nuclear import of the influenza virus genomic RNA in permeabilized Buffalo rat liver cells has recently been shown to occur only in the presence of the viral nucleoprotein (NP), which coats the viral RNAs (33). Requirements for nuclear import of the influenza protein–RNA complex are indistinguishable from those for NLS-containing proteins, indicating import of this viral nucleic acid is mediated by the associated protein (32). Thus, the data presented here may have relevance to other basic and applied processes such as nuclear import of either endogenous or pathogen-derived protein–nucleic acid complexes, as well as delivery of foreign genes into the plant cell nucleus. In addition, the data support the use of protein nuclear import inhibitors such as WGA and nonhydrolyzable GTP analogs in the study of nuclear import processes in plants.

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