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Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco

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ABSTRACT **Previously, we have shown that *Agrobacterium*–plant cell transferred DNA (T-DNA) transport into the host cell nucleus is likely mediated by two specific bacterial proteins, VirD2 and VirE2. Here, we used these proteins to study molecular pathways of nuclear import. First, the role of VirE2 nuclear localization signals (NLSs) in the T-DNA transport pathway was examined by using tobacco plants transgenic for deletion mutants of VirE2. In these plants, the virulence of wild-type *Agrobacterium* was reduced possibly by competition for the cellular nuclear import machinery. Second, we analyzed the nuclear localization of VirE2 and VirD2 in the nonhost monocot maize. Part of the known recalcitrance of monocots to transformation by *Agrobacterium* could be due to a potential selectivity in nuclear import pathways in monocotyledonous and dicotyledonous plants. Nuclear transport of VirD2 and VirE2 in maize leaves and roots was compared to that in tobacco protoplasts and roots. Both proteins accumulated in maize leaf and tobacco protoplast nuclei as well as in nuclei of immature root cells. In contrast, VirD2 and VirE2 expressed in mature roots of maize and tobacco remained cytoplasmic. Point mutations of VirE2 nuclear localization signals, NSE 1 and NSE 2, also revealed that, in maize, the NSE 1 signal was mainly responsible for nuclear import; in contrast, both signals functioned independently in tobacco protoplasts.**

In plant–pathogen interactions, the invading microorganism often adapts the existing cellular machinery for its own needs. Therefore, nuclear import of *Agrobacterium* transferred DNA (T-DNA) and its associated proteins provides a unique and useful experimental system to study nuclear transport of proteins and nucleic acids in plants. Recently, several studies have begun to examine protein import into plant nuclei (reviewed in ref. 1). These studies demonstrate that plant nuclear uptake requires specific nuclear localization signals (NLSs) that are homologous to those described for animal systems (2–6). The mechanisms by which these NLSs operate in plants are unknown. For example, within a single plant, can NLSs compete for the same import pathway or are some NLSs active only in certain tissues? Do some NLSs function specifically in monocotyledonous or dicotyledonous plants? The potentially selective activity of different NLSs may represent an important regulatory mechanism for control of both gene expression and interactions with plant pathogens. To examine (*i*) potential competition between NLSs and (*ii*) differences in nuclear import pathways between dicots and monocots, we used two *Agrobacterium* proteins proposed to be involved in T-DNA nuclear transport.

The interaction of *Agrobacterium* with plant cells is the only known natural example of interkingdom DNA transfer. Most functions for *Agrobacterium*–plant cell DNA transfer are carried on a large (200 kb) Ti (tumor-inducing) plasmid contained in the bacterial cell. There are two important genetic components on the Ti plasmid. One component, the T-DNA, is copied and transferred to the plant cell as a single-stranded DNA (ssDNA) molecule, the T-strand (reviewed in refs. 7 and 8). The second component of the Ti plasmid, the virulence (*vir*) region, provides most of the trans-acting products for T-DNA transfer.

Evidence to date suggests that the T-strand directly associates with two different protein products of the *vir* region. During T-strand synthesis, the VirD2 protein tightly (probably covalently) attaches to the 5′ end of the T-strand molecule (9–12), while VirE2, a ssDNA-binding protein (SSB), is proposed to coat the T-strand along its entire length (13–15). Binding of VirE2 to ssDNA is cooperative and results in formation of long, unfolded, and very thin (<2-nm diameter) protein–ssDNA complexes (16). The T-strand with its associated proteins, VirD2 and VirE2, comprise the *Agrobacterium* T-DNA transfer complex, designated the "T-complex" (17, 18).

To genetically transform the host plant cell, the T-complex ultimately must reach the host cell nucleus. We and others have recently demonstrated nuclear localization of VirD2 in tobacco cells (4, 19) and have identified a bipartite NLS at the carboxyl terminus of the protein (4). These results suggest that the VirD2 protein, attached to the 5′ end of the T-strand, acts to direct the T-complex in a polar fashion to the host cell nucleus. However, that deletion of VirD2 NLS decreases but does not completely abolish tumorigenicity (20, 21) indicates that VirD2 is not the sole mediator of the T-complex nuclear uptake. Indeed, in tobacco, VirE2 was also identified as a nuclear-localizing protein that contains two bipartite NLS sequences designated NSE 1 and NSE 2 (3). That VirE2 is involved in nuclear uptake of T-DNA is strengthened by the observation that tobacco plants transgenic for VirE2 complement the virulence of an *Agrobacterium* strain with an inactivated *virE* locus (3) and indicates that VirE2 functions inside the plant cell.

Here we used two approaches to study the nuclear-localizing function of VirD2 and VirE2. First, the role of VirE2 in nuclear import pathway was studied by using transgenic tobacco plants that express deletion mutants of this protein. The results suggested that the endogenous VirE2 mutant protein inhibited nuclear import of the invading T-complex, reducing *Agrobacterium* virulence. Second, the function of VirE2 and VirD2 proteins was assayed in a nonhost monocot, maize, and in a host dicot, tobacco. In these experiments, VirD2 and VirE2 localized to the cell nuclei of maize leaves, tobacco protoplasts, and maize and tobacco immature root tissue. In contrast, both proteins remained cytoplasmic when expressed in the mature maize or tobacco roots.

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Abbreviations: GUS, β-glucuronidase; NLS, nuclear localization signal; ssDNA, single-stranded DNA; SSB, ssDNA-binding protein; T-DNA, transferred DNA; T antigen, tumor antigen; TMV, tobacco mosaic virus.

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MATERIALS AND METHODS

Plasmids. To monitor subcellular localization, β-glucuronidase (GUS) and fusion proteins GUS-VirD2 and GUS-VirE2 were expressed from pRTL2GUS (22), pGD-A (4), and pGUSE2 (3), respectively. Oligonucleotide-directed mutagenesis of pGUSE2 was used to construct GUS-nsb-1 and GUS-nsb-2 mutants (23). All mutants were verified by dideoxynucleotide sequencing (24).

Transformation of Plant Tissues and Protoplasts and GUS Assay. Tobacco (*Nicotiana tabacum* cv. Turk) and A188 maize seedlings were germinated *in vitro* on H_2O-agar. Upper parts of the root were used as a source of mature root tissue, while root tips represented immature root tissue. Tissue segments 0.5–1.0 cm long were removed and placed on MS-agar plates (25). By using the Biolistic™ particle delivery system (26), ≈2 μg of plasmid DNA was delivered into leaf and root epidermal cell layers. GUS histochemical staining (27) was performed 24 hr after bombardment. Tissues were incubated in the staining solution for 2–4 hr at 37°C. Tobacco protoplasts were isolated, transformed with plasmid DNA, and assayed for GUS activity as described (3).

VirE2 Expression and Purification. VirE2 was expressed from p118E2 plasmid (3). The nsb-1 and nsb-2 mutants were produced by oligonucleotide-directed mutagenesis of p118E2 (23). VirE2 and its mutants were overproduced in BL21(DE3) strain of *Escherichia coli* (28) and purified as described (14, 16).

Gel Mobility-Shift Assay. A 75-mer oligonucleotide [nucleotides 5632–5708 of tobacco mosaic virus (TMV) cDNA (U1 strain; ref. 29)] was end-labeled by phosphorylation with T4 polynucleotide kinase (30) to a specific activity of 10^6 cpm/μg. This probe (10 ng) was incubated for 15 min at room temperature with the indicated amounts of protein. After incubation, the reaction mixtures were analyzed as described (29).

VirE2 Transgenic Plants and *Agrobacterium* Infection. All transgenic tobacco plants (*Nicotiana tabacum* cv. Turk) were produced as described (3). Expression of VirE2, VirE2-del B, VirE2-del C, VirE2-del D, and TMV P30 proteins in these plants was verified by immunoblot (Western blot) analysis of tissue extracts (3). VirE2-del B (deleted amino acid residues 341–556), VirE2-del C (deleted residues 228–244), and VirE2-del D (deleted residues 296–310) were produced as described (3).

RESULTS

Inhibition of *Agrobacterium* Virulence by Expression of VirE2 Deletion Mutants in Transgenic Tobacco Plants. Since VirE2 is likely involved in the T-complex nuclear import, it may be possible to construct VirE2 mutants that, when expressed in transgenic plants, will interfere with the wild-type VirE2 molecules present on the invading T-complex during infection. This interference may then inhibit *Agrobacterium* tumorigenicity. To test this idea, three VirE2 mutations were expressed in transgenic plants. VirE2-del B lacks 215 carboxyl-terminal amino acids; this mutant is unable to bind ssDNA, but it retains both NSE signals (3). The VirE2-del C and VirE2-del D mutations specifically remove NSE 1 or NSE 2, respectively (3); ssDNA binding activity of these mutants is attenuated (VirE2-del C) or blocked (VirE2-del D) (Table 1). These transgenic tobacco plants were inoculated with *Agrobacterium* pTiA6/pSM219 strain. This strain is an insertional mutant of the *pinF* locus; *pinF* products generally are not required for virulence, and therefore pTiA6/pSM219 has wild-type tumorigenicity (32).

All wild-type plants developed tumors when inoculated with the pTiA6/pSM219 strain. In contrast, the virulence of this *Agrobacterium* strain was significantly reduced in plants expressing VirE2-del B and VirE2-del D mutants. Furthermore, even expression of the wild-type VirE2 in transgenic plants reduced the tumorigenicity of pTiA6/pSM219. VirE2-del C inhibitory effect was comparable to that of the wild-type VirE2 (Table 1). Tobacco plants transgenic for an unrelated SSB protein, P30 of TMV (31), were used (*i*) to test whether another SSB that does not have functional NLSs could interfere with *Agrobacterium* virulence and (*ii*) to control whether transgenic plants are susceptible to subsequent infection by *Agrobacterium*. Similarly to wild-type tobacco, P30 transgenic plants were efficiently infected by the pTiA6/pSM219 strain (Table 1 and ref. 3). These results indicate that VirE2 expressed in transgenic plants may specifically interfere with nuclear import of the wild-type T-complex during infection.

Table 1. Effect of VirE2 transgenic tobacco plants on *Agrobacterium* virulence

Host *Nicotiana tabacum*	Tumor formation, % of inoculated plants	NLS NSE1	NLS NSE2	SSB
Wild-type	100	NA	NA	NA
Transgenic for				
VirE2	75	+	+	+
VirE2-del B	33	+	+	−
VirE2-del C	70	−	+	±
VirE2-del D	55	+	−	−
TMV P30	100	NA	NA	+

Eight to 10 individual transgenic plants were inoculated in duplicate with *Agrobacterium* strain pTiA6/pSM219 as described (3). Crown gall tumors were scored 2 weeks after inoculation. Data are expressed as the percentage of plants that developed tumors of the total number of inoculated plants. NLS columns indicate the presence of VirE2 NLS sequences NSE1 and NSE2 in the mutant proteins (3). SSB–ssDNA binding activity of the VirE2 proteins (3) and of the P30 protein of TMV (31) is shown. NA, not applicable.

Nuclear Localization of VirD2 and VirE2 in Maize and Tobacco. Next, the nuclear import pathway used by VirE2 and VirD2 and, presumably, the entire T-complex was characterized in the host (dicot) and nonhost (monocot) plants. Although monocots are recalcitrant to *Agrobacterium* infection (33), the T-complex nuclear import in these plants may still occur. Thus, we assayed VirE2 and VirD2 for their ability to specifically accumulate in the cell nuclei of the monocot maize. To monitor subcellular localization, VirD2 and VirE2 were transiently expressed in the epidermal cells of maize leaves as carboxyl-terminal fusion proteins with a reporter enzyme, GUS (3, 4). Histochemical staining revealed specific accumulation of GUS-VirD2 and GUS-VirE2 in the cell nuclei (Fig. 1 *B* and *C*). In control experiments with GUS alone, nuclear accumulation of the GUS product was not detected (Fig. 1*A*). Then, nuclear import of GUS-VirD2 and GUS-VirE2 was examined at two different developmental stages of maize root tissue—immature root tips and mature fully developed basal parts of the root. Both GUS-VirD2 and GUS-VirE2 were imported into the nuclei of immature root cells (Fig. 1 *F* and *G*). Surprisingly, however, these proteins did not accumulate in the cell nuclei of mature roots (Fig. 1 *I* and *J*). Note that the micrographs in Fig. 1 show unfixed whole organs; consequently, their resolution cannot be compared to fixed tissue sections. As biolistic DNA delivery results in a low frequency of transformed cells, it was necessary to examine entire target organs for GUS expression. To facilitate identification of the cell nucleus, nuclear-specific staining of maize leaf tissue with acridine orange is shown in Fig. 1*D*. The spherical pattern of the acridine orange staining (Fig. 1*D*) corresponds to the blue staining of cell nuclei that accumulate the GUS product (Fig. 1*B*).

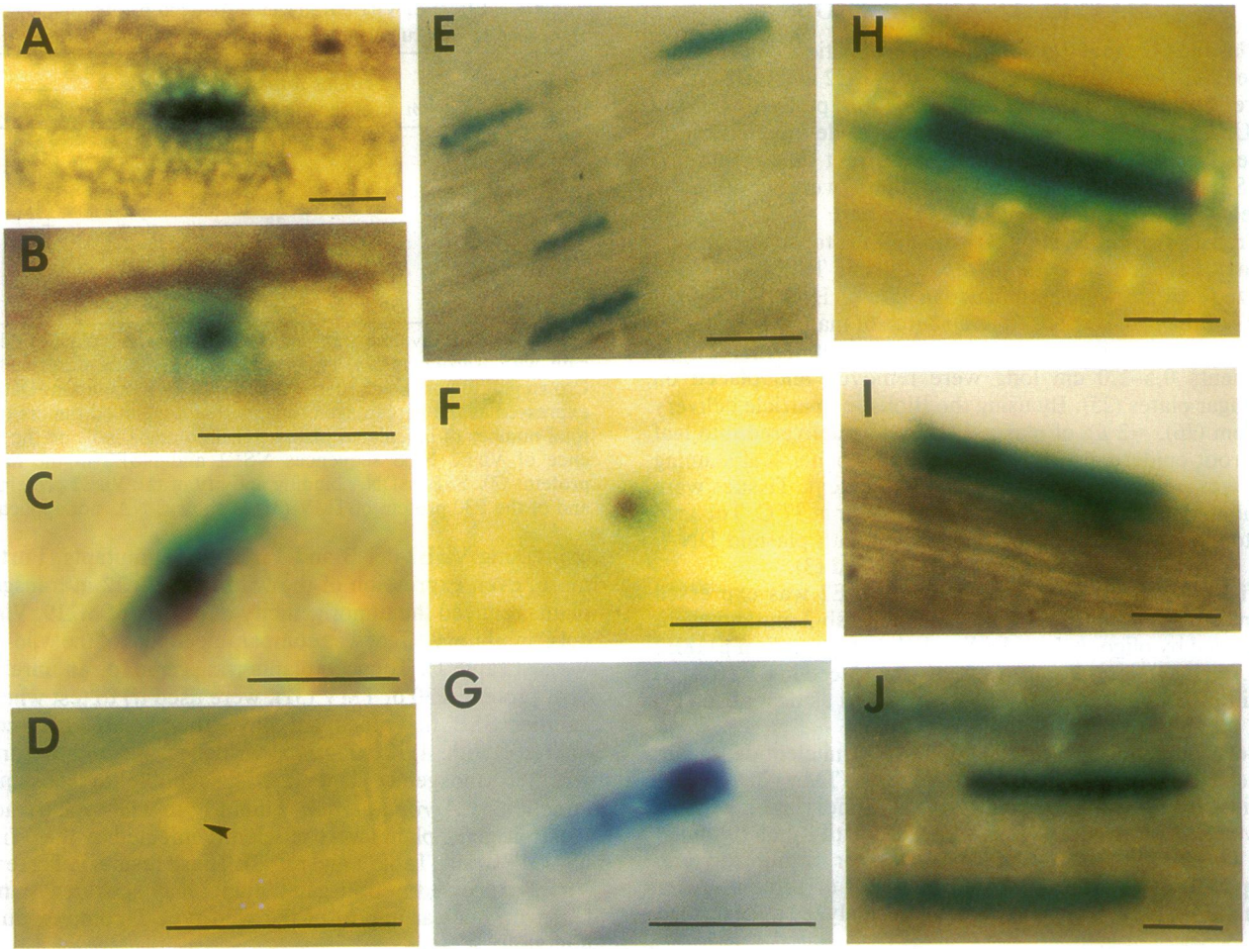

FIG. 1. Subcellular localization of GUS, GUS-VirD2, and GUS-VirE2 in maize leaves (*A*, *B*, and *C*) and in immature (*E*, *F*, and *G*) and mature (*H*, *I*, and *J*) roots. (*A*, *E*, and *H*) GUS alone. (*B*, *F*, and *I*) GUS-VirD2. (*C*, *G*, and *J*) GUS-VirE2. (*D*) Nuclear-specific staining of maize leaves with acridine orange; arrow indicates the stained nucleus. Note that blue rectangular epidermal cells represent cytoplasmic GUS staining, while the spherical pattern of the blue corresponds to nuclear staining. (Bar = 20 μm.)

The levels of GUS activity were quantified spectroscopically by measuring the intensity of the indigo dye in the nucleus and cytoplasm of individual cells (3, 4), where nuclear localization efficiency of the GUS-VirD2 was defined as 100% and nuclear localization of GUS alone was defined as zero. Table 2 shows that, in maize leaves and immature roots, nuclear accumulation of VirE2 was less efficient than that of VirD2. While all of the GUS-VirD2 product concentrated in the nuclei, ≈30% of GUS-VirE2 product remained in the cytoplasm (Table 2). This quantitative study agrees with the micrographs shown in Fig. 1; nuclear localization with GUS-Vir D2 shows spherical staining corresponding to the cell nucleus, whereas GUS-VirE2 localization is more diffuse, showing both nuclear and cytoplasmic staining (compare Fig. 1 *B* and *F* to Fig. 1 *C* and *G*).

In the dicot host plant, tobacco, both GUS-VirD2 and GUS-VirE2 accumulated to high levels in the nuclei of immature root cells (100% and 89%, respectively) and cultured protoplasts (100% and 98%, respectively) (Table 2). Neither GUS-VirD2 nor GUS-VirE2 were detected in the cell nuclei of mature maize roots (Fig. 1 *I* and *J*) or mature tobacco roots (Table 2). These results suggest (*i*) that *Agrobacterium* VirD2 and VirE2 proteins function in dicots and monocots and, importantly, (*ii*) that nuclear uptake of these

Table 2. Quantitation of nuclear and cytoplasmic levels of GUS activity

	GUS activity, % of maximal											
	Maize						Tobacco					
	Leaves		Immature roots		Mature roots		Protoplasts		Immature roots		Mature roots	
Construct	N	C	N	C	N	C	N	C	N	C	N	C
GUS	0	100 (8)	0	100 (8)	0	100 (5)	0	100 (6)	0	100 (9)	0	100 (9)
GUS-VirD2	100 (5)	0	95 (7)	5 (5)	0	100 (5)	100 (6)	0	100 (9)	0	0	100 (5)
GUS-VirE2	73 (5)	27 (5)	70 (7)	30 (7)	0	100 (7)	98 (8)	2 (2)	89 (7)	11 (7)	0	100 (8)
GUS-nsb-1	0	100 (2)	ND	ND	ND	ND	76 (5)	24 (5)	ND	ND	ND	ND
GUS-nsb-2	45 (3)	55 (3)	ND	ND	ND	ND	64 (3)	36 (3)	ND	ND	ND	ND

The intensity of indigo dye precipitates formed during the GUS assay in the plant cell cytoplasm (columns C) and nucleus (columns N) was quantified by photodensitometry measuring the specific light transmission of the indigo GUS product in photographic negatives of single cells (4). Nuclear localization of GUS-VirD2 was defined as 100% activity, and GUS alone was defined as 0% activity. Each result is a mean value of independent measurements of 3–10 different cells; SEM values are in parentheses. ND, not done.

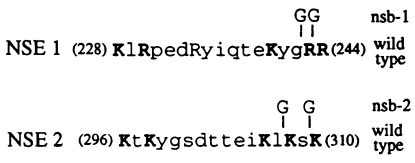

FIG. 2. NSE 1 and NSE 2 sequences of VirE2. Amino acid sequences are shown in one letter code; specific amino acid substitutions are shown above the corresponding residue in the wild-type sequence. All basic residues are in uppercase letters; basic amino acids of the two domains of the bipartite NSE signals are in boldface letters.

proteins depends on the developmental stage of the target tissue both in the host (tobacco) and in nonhost plants (maize).

Point Mutation Analysis of VirE2 Function in Maize and Tobacco. We further examined VirE2 function in maize and tobacco, using specific amino acid substitutions in its NLS signals. Two substitution mutants, nsb-1 and nsb-2, were constructed; nsb-1 targeted two carboxyl-terminal arginines of NSE 1, while nsb-2 substituted two carboxyl-terminal lysines of NSE 2 (Fig. 2); in the case of nucleoplasmin, a paradigm for a bipartite NLS, this carboxyl-terminal basic region is critical for the NLS activity of the protein (34). Table 2 shows that in maize leaves mutation of Lys-308 and Lys-310 to glycine (nsb-2) decreased but did not block nuclear uptake. Nuclear accumulation of GUS-nsb-2 was lower than that of the wild-type GUS-VirE2 (45% vs. 73%, respectively). In contrast, replacement of Arg-243 and Arg-244 with glycine residues (nsb-1) completely inhibited the nuclear import of GUS-nsb-1 (Table 2). When nsb-1 and nsb-2 were expressed in tobacco protoplasts, the mutant proteins were found both in the cytoplasm (24% and 36%, respectively) and in the cell nucleus (76% and 64%, respectively) (Table 2). These results indicate that in maize leaves the nuclear import of VirE2 is mediated predominantly by the NSE 1 sequence. In tobacco, however, both NSE1 and NSE2 are independently active (see also ref. 3).

Since both VirE2 NLSs overlap its ssDNA binding domain (3), the effect of amino acid substitutions in nsb-1 and nsb-2 on VirE2 ssDNA binding was examined. nsb-1 and nsb-2 mutants (not fused to GUS) were overproduced in *E. coli*, purified, and tested for ssDNA binding. Fig. 3 shows that full-length VirE2-bound ssDNA in a cooperative fashion (Fig. 3 and ref. 16). Characteristic of binding cooperativity (16, 35), subsaturating amounts (10–15 ng) of VirE2 caused a significant retardation of the protein-bound ssDNA; nsb-1 bound ssDNA in a distinct "ladder" pattern, which is characteristic of low cooperative binding (35). At subsaturating concentrations of nsb-1, ssDNA molecules were not fully coated with the protein, resulting in formation of discrete protein–ssDNA complexes with decreasing electrophoretic mobility (Fig. 3). Thus, uncharged replacements of Arg-243 and Arg-244 in nsb-1 interfere with protein–protein interactions required for binding cooperativity. Unlike nsb-1, the nsb-2 mutant of VirE2 was completely unable to bind ssDNA (Fig. 3); however, nuclear localization of nsb-2 was only decreased (Table 2) but not blocked. This observation suggests that the two functions, nuclear localization and ssDNA binding, are separable.

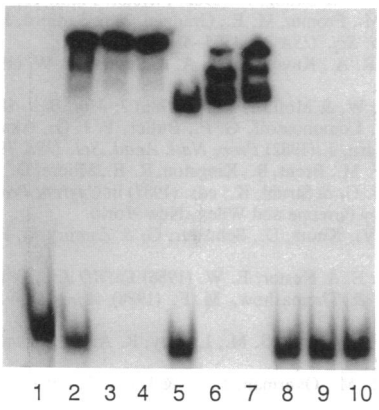

FIG. 3. ssDNA binding activity of the nsb-1 and nsb-2 mutants of VirE2. Lanes: 1, ssDNA probe alone; 2, 0.1 μg of VirE2; 3, 0.2 μg of VirE2; 4, 0.5 μg of VirE2; 5, 0.1 μg of nsb-1; 6, 0.2 μg of nsb-1; 7, 0.5 μg of nsb-1; 8, 0.1 μg of nsb-2; 9, 0.2 μg of nsb-2; 10, 0.5 μg of nsb-2.

DISCUSSION

This work had two specific goals: (*i*) to further characterize the function of VirE2 in T-complex nuclear import by using transgenic plants, and (*ii*) to compare nuclear import pathways for VirE2 and VirD2 in monocots and dicots. The role of VirE2 nuclear import was studied *in vivo* by using a variation of a "dominant negative mutation" strategy. This approach, traditionally used in studies of gene function (36), involves expression of a mutated gene that represses the activity of the wild-type gene. In the case of VirE2, its deletion mutants were constitutively expressed in transgenic host plants; when inoculated with *Agrobacterium*, all of these plants displayed different levels of resistance to the infection. Three scenarios may be suggested to explain these results. (*i*) Some mutations may produce an altered protein conformation, leading to an irreversible interaction between the mutant proteins and cellular factors involved in nuclear import. In this case, the inhibitory effect of these mutants is expected to be high; VirE2-del B and VirE2-del D mutants, which produced the strongest resistance to *Agrobacterium* infection, may act by this mechanism. (*ii*) Wild-type VirE2 protein expressed in transgenic plants may be partially sequestered by binding to cellular RNAs. However mutants such as VirE2-del B and VirE2-del D that do not bind single-stranded nucleic acids may increase the level of free mutant protein, resulting in a more efficient inhibition of nuclear import. (*iii*) Finally, expression of wild-type VirE2 or more functional mutant derivatives such as VirE2-del C may interact reversibly and, consequently, may compete less efficiently with the invading T-complex for cellular factors.

Although mutant VirE2 proteins expressed in transgenic plants reduce nuclear import of the T-complex, these plants are viable; thus, their normal cellular processes of nuclear transport are not significantly compromised. This result may indicate that not all pathways for nuclear import are being competitively blocked. Similar observations have been reported for competitive inhibition of nuclear import in animal systems. For example, mutants of simian virus 40 large tumor (T) antigen and ICP4 protein of herpes simplex virus have been shown to inhibit nuclear import of the wild-type T antigen and ICP4 and some (but not all) other karyophilic proteins, suggesting that the observed interference involved only few of the existing pathways for nuclear import (reviewed in ref. 37).

Next, we determined if a nonhost plant, the monocot maize, possesses a nuclear import pathway that can transport VirE2 and VirD2. While a monocot transcription factor, the Opaque-2 protein of maize, has been shown to localize to dicot nuclei (38), it is unknown if karyophilic proteins normally active in dicots can also function in monocotyledonous plants. Our results with *Agrobacterium* VirD2 and VirE2 proteins suggest that there is no simple answer to this question. Both VirD2 and VirE2 clearly accumulate in maize leaf nuclei. However, while nuclear accumulation levels of VirD2 and VirE2 in tobacco protoplasts are quantitatively

similar, in maize VirD2 nuclear import is more efficient than that of VirE2. In addition, the two NLS signals of VirE2, NSE 1 and NSE 2, function independently in tobacco protoplasts. In contrast, only NSE 1 is independently active in maize leaves.

Nuclear localization of VirD2 and VirE2 may be developmentally regulated. Both proteins accumulated in the nuclei of tobacco protoplasts, maize leaf epidermal cells, and tobacco and maize immature root epidermis. In mature root epidermis of tobacco or maize seedlings, however, VirD2 and VirE2 remained cytoplasmic. That the GUS-VirD2 and GUS-VirE2 fusion proteins were produced from transfected DNA implies that the plasmid DNA entered the nucleus and was transcribed, and the pre-mRNA was exported from the nucleus into the cytoplasm. However, GUS-VirD2 and GUS-VirE2 themselves were not imported into the nucleus, suggesting that some pathway(s) for nuclear transport—e.g., the T-complex import pathway—are repressed in mature root tissue.

The mechanism by which some NLSs can be active in developing tissue and remain nonfunctional in mature tissue is unknown. One possibility is that VirD2 and VirE2 nuclear import is mediated by specific cellular NLS-binding proteins (reviewed in ref. 39) that may be absent in fully developed root epidermis. Potentially, this selective nuclear transport may reflect a regulatory mechanism for developmentally-specific gene expression; for example, only a subset of transcription factors may reach the cell nucleus at different stages of tissue development. If true, *Agrobacterium* VirD2 and VirE2 proteins may be useful to study differential regulation of nuclear import in plants.

The VirE2 nuclear import in maize leaves was reduced compared with that of VirD2. To better characterize the VirE2 nuclear uptake in monocots, point mutagenesis was used for functional study of VirE2 NLSs. Previously, we identified two NLSs, termed NSE 1 and NSE 2, in the VirE2 protein. NSE 1 and NSE 2 were characterized as bipartite-type signals (3). In nucleoplasmin, point mutagenesis of the carboxyl-terminal basic domain of its bipartite NLS demonstrated that although substitutions of single residues did not block nuclear localization, simultaneous substitution of at least two of the basic residues in this domain resulted in a substantial decrease in nuclear uptake (34). In VirE2, uncharged substitutions of the two conserved carboxyl-terminal basic residues in NSE 2 decreased but did not eliminate nuclear accumulation in maize leaves. In contrast, a similar mutation in the NSE 1 sequence completely blocked VirE2 nuclear import. These observations indicate that NSE 1 may be mainly responsible for nuclear localization of VirE2 in maize leaves. This is different from tobacco, where both NSE sequences function equally in the VirE2 nuclear transport (3). Furthermore, these mutations also altered ssDNA binding of VirE2. The two point mutations in the NSE 2 region were sufficient to block VirE2 binding to ssDNA; conversely, the point mutations in NSE 1 resulted in an attenuated (noncooperative) ssDNA binding.

Our results indicate that VirE2 is imported into the host plant cell nucleus by a pathway that may be inhibited by VirE2 mutants expressed in transgenic plants. Furthermore, nuclear import pathways for *Agrobacterium* VirE2 and VirD2 proteins exist in both host dicot (tobacco) and nonhost monocot (maize) plants. Interestingly, nuclear import of VirE2 and VirD2 in both maize and tobacco is dependent on the developmental stage of the tissue. Similarly, while both VirD2 and VirE2 NLSs share homology with many other NLS signals, not all NLSs can function in nuclear transport of the T-complex. For example, VirD2 NLS can be substituted with the NLS of the tobacco etch virus NIa protein; however, substitution with the simian virus 40 large T antigen NLS [which itself is active in plants (5)] blocks the virulence of *Agrobacterium* (21). Potentially, these different pathways of nuclear transport may function to independently control traffic of diverse substrates through the same nuclear pore. Our data suggest that the nuclear import of *Agrobacterium* T-DNA and its associated proteins may serve as a model system to study different pathways for nuclear transport of proteins and protein–nucleic acid complexes in plant cells.

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