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The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals

(*Agrobacterium*/yeast/plant protoplast/immunofluorescence)

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ABSTRACT **_Agrobacterium tumefaciens_ causes neoplastic growth in plants by transferring a piece of DNA, called T-DNA, into the nucleus of the plant cell. The virulence protein VirD2 of _A. tumefaciens_ is tightly linked to the T-DNA and is thought to direct it to the plant genome. Here we show that the VirD2 protein contains two nuclear localization signals that are functional both in yeast and in plant cells. One signal is located in the N-terminal part of the protein and resembles a single-cluster-type nuclear localization signal. The second signal is near the C terminus and is a bipartite-type nuclear localization signal. The involvement of these sequences in the entry of the T-DNA into the nucleus is discussed.**

Agrobacterium tumefaciens, the causative agent of crown gall disease, transfers into plant cells genes coding for enzymes involved in the synthesis of plant growth factors. These genes are carried by the T-DNA, a well-defined region of a large plasmid called Ti (*t*umor-*i*nducing). The T-DNA is delimited at both extremities by two almost perfect 25-base-pair repeats, or border sequences (for reviews see refs. 1–3). In the presence of wounded plant cells the T-DNA is liberated from the Ti plasmid and is transferred to the plant cell, where it finally enters the nucleus and integrates into the plant genome. Proteins encoded by the virulence region of the Ti plasmid, essential for tumorigenicity, mediate the mobilization of the T-DNA and probably assist in its integration. Two virulence proteins have been shown to form a complex with the T-DNA. The VirE2 protein, a single-stranded DNA-binding protein, covers free single-stranded T-DNA (T-strand) within the bacterium (4). Upon cutting at the T-DNA borders the VirD2 protein attaches (most likely covalently) to the 5′ end of the processed T-DNA (5–8). Therefore, it has been proposed to be transported into the plant cell together with the T-DNA. VirD2 may thus perform several functions within the plant cell: protect the T-DNA against nucleases, target it into the plant nucleus, and integrate it into the plant genome (8–10). Here we study the property of VirD2 in nuclear targeting.

Entry of proteins into the nucleus is a selective process that requires the activation of the nuclear pore complex (refs. 11 and 12; for review see refs. 13 and 14). This activation is mediated by a nuclear localization signal (NLS) carried either by the transported protein itself or by a helper protein (15). Two types of NLS have been described. The first consists of a single cluster of positively charged amino acids, the consensus sequence being K-R/K-X-R/K (16). The second type is a bipartite signal in which two necessary sequence elements made up of basic amino acids are separated by about 10 undefined amino acids (17, 18). The simian virus 40 (SV40) large tumor (T)-antigen and the nucleoplasmin NLSs are examples of these two types. Inspection of the 424 amino acids of VirD2 reveals two regions with similarities to such sequences (19). One is located in the N-terminal part of the protein and resembles the SV40 T-antigen or single-cluster-type signal (Fig. 1*a*). The other is in the C terminal part and belongs to the nucleoplasmin or bipartite-type family of signals (Fig. 1*b*). Interestingly, the sequence corresponding to the C-terminal putative NLS is perfectly maintained in different *Agrobacterium* strains although it belongs to the C-terminal half of VirD2, which is <20% conserved (see Fig. 1*b*), whereas the N-terminal part is >80% conserved (refs. 19 and 20; see Fig. 2*a*). Howard *et al.* (21) showed recently that the C-terminal signal is the only one having nuclear targeting properties, whereas Herrera-Estrella and coworkers (19) previously found that the N-terminal 292 amino acids of VirD2 contained such a property.

Plant nuclear proteins contain sequences that are similar to the NLSs described for other eukaryotic organisms (22, 23). However, these sequences have not been extensively characterized. Defined NLS sequences from nonplant proteins are correctly recognized in different organisms; the SV40 T-antigen NLS has been successfully tested in mammalian cells (24), in yeast cells (25, 26), and in plant cells (27), and a yeast nuclear protein has been shown to enter the nucleus of a higher eukaryotic organism (P. Wagner and M.N.H., unpublished work). Thus, the recognition system involved in nuclear import is most likely universal. Our strategy was therefore to test the putative VirD2 NLS sequences in yeast cells and then to assay defined segments of VirD2 in plant cells. We found that VirD2 has two nuclear import signals and that yeast can indeed be used to test heterologous NLSs.

MATERIALS AND METHODS

Strains, Plasmids, and Media. *Escherichia coli* strain NM522 (28) was used for cloning, and the yeast strain JK9-3d *a/α, leu2/leu2, ura3/ura3, rme1/rme1, trp1/trp1, his4/his4*, HMLa/HMLa was used for immunofluorescence experiments. The yeast vector pMBL214 (29) is a 2-μm shuttle plasmid containing the β-lactamase gene and the *LEU2*d gene as markers for selection in *E. coli* and in yeast, respectively. pMBL214 also contains a *lacZ* gene controlled by the galactose-inducible promoter pGal7. The *lacZ* coding sequence is preceded by an out-of-frame start codon. Cloning into the unique-*Sma* I site between the start codon and *lacZ* allows proper alignment of the start codon. Bacterial (LB) and yeast (YNB supplemented with the required amino acids) media were prepared as described (28, 30). Plasmid pMY1153 contains the *virD* region of the octopine Ti plasmid pTiA6NC (31). pTTO is a pUC derivative containing the promoter and the terminator for the 35S RNA of cauliflower mosaic virus (CaMV) (32).

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Abbreviations: CaMV, cauliflower mosaic virus; DAPI, 4′,6-diamino-2-phenylindole; FITC, fluorescein-5-isothiocyanate; NLS, nuclear localization signal; SV40, simian virus 40.

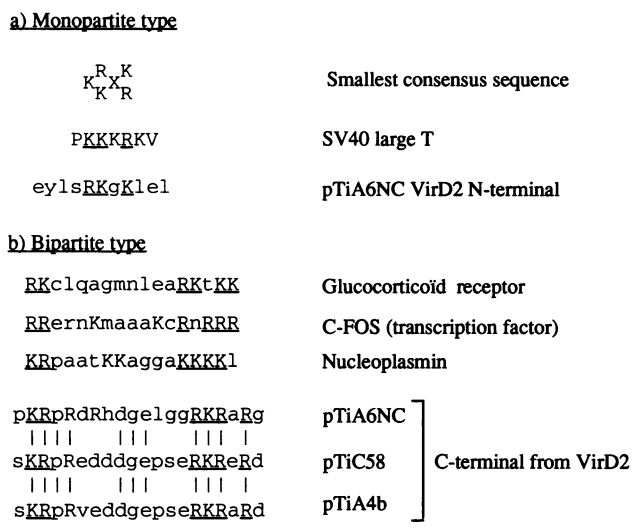

FIG. 1. Sequence comparison of monopartite (*a*) and bipartite (*b*) NLSs (references for bipartite NLSs are in ref. 18). Amino acids considered to be involved in nuclear targeting are underlined. The homologies between three VirD2 C-terminal NLSs are indicated by vertical bars.

Constructions. *For analysis in yeast.* Various *virD2–lacZ* fusions were constructed by using the *Sma* I site of pMBL214. The *virD2* gene, and deletion derivatives of it, were amplified by PCR from the plasmid pMY1153 (31) by using the following primers in three different combinations (Fig. 2*a*): pr1, 5′-GGG-ACG-CGC-ACC-CGG-GGG-*ATG-CCC-GAT-CGC-GCT-CAA-GTA-ACA-TTC*-3′ (intact N terminus); pr2, 5′-CAG-CGT-TCA-GCC-CGG-GAT-CTC-GAT-*ATG-CCC-GTT-CCG-CCG-GAT-CAA-ATC*-3′ (deleted N terminus); pr3, 5′-CTT-TGA-TAT-CTA-CCC-GGG-*CCC-GCG-CCC-ATC-GTC-GCG-ACG-ATT-ACC*-3′ (intact C terminus); pr4, 5′-CAC-GCG-GAC-GCC-CGG-*GGA-GAA-GCT-TCC-GGT-TGC-TCG-GTA-CCG-ATG*-3′ (deleted C terminus). All primers contained an extra *Sma* I site (underlined). The part of each primer corresponding in sequence to *virD2* is shown in italics. After cleavage by *Sma* I, the PCR products were cloned into the *Sma* I site of pMBL214, resulting in the plasmids pVD2 (primers pr1 and pr3), pVD2.2 (primers pr1 and pr4), and pVD2.3 (primers pr2 and pr3) (Fig. 2*a*). The N-terminal primers contribute their own ATG codons to restore the *lacZ* reading frame. The oligonucleotides corresponding to the putative isolated N-terminal NLS (5′-GG-GAG-TAC-CTG-TCC-CGT-AAG-CTG-GAA-CTC-CCC-3′) and C-terminal NLS (5′-GG-CCA-AAG-CGT-CCG-CGT-GAC-CGT-CAC-GAT-GGA-GAA-TTG-GGT-GGA-CGC-AAA-CGT-GCA-AGA-GGC-CCC-3′) were annealed to their complementary oligonucleotides and cloned directly into the *Sma* I site of pMBL214. This resulted in restoration of the frame between the start codon from the plasmid pMBL214 and the *lacZ* sequence. The resulting plasmids are called pVDN and pVDC, respectively (Fig. 2*b*). pVCO, a construct containing the oligonucleotide of the N-terminal NLS inserted in the wrong orientation, was used as a negative control. All constructs in pMBL214 were tested in yeast for β-galactosidase activity on medium containing galactose and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

Constructions for analysis in Nicotiana plumbaginifolia. A fragment containing the DNA sequence of the plant promoter and terminator signals for the 35S RNA of CaMV was subcloned from the plasmid pTTO (32) into the *Eco*RI/*Hin*dIII sites of the plasmid pBR328 to give pBR35S. The NLS–*lacZ* derivatives of pVDN, pVDC, and pVCO were amplified by PCR using oligonucleotides homologous to the pMBL214 polylinker (pr5, 5′-CTT-GTA-ATA-CTA-GTG-*ATC-CAC-TGC-AGG-TCG-ACG-GAT-CCG-GGG*-3′) and to the *lacZ* 3′ end (pr6, 5′-TTA-TCG-AAC-TAG-TCC-*TTT-TTG-ACA-CCA-GAC-CAA-CTG-GTA-ATG-GTA GCG*-3′) (Fig. 2*c*). Each primer also contained a recognition sequence for *Spe* I (ACTAGT, underlined; the identity with pMBL214 polylinker or *lacZ* 3′ end is in italics). The amplified fragments were digested with *Spe* I and inserted into the *Spe* I site of pBR35S, resulting in pBR35SN, pBR35SC, and pBR35SCO. Constructs pVDN, pVDC, pVCO, pBR35SN, pBR35SC, and pBR35SCO were checked by sequence analysis at the fusion sites (sequencing kit from USB).

Transformation. *E. coli* and yeast transformations were performed according to published procedures (30, 33). *N. plumbaginifolia* protoplasts were transfected with 20 μg of DNA per 3×10^5 protoplasts by the PEG method (34).

Indirect Immunofluorescence. *Yeast.* Yeast cells (40 ml) were grown in the minimal medium YNB supplemented with tryptophan, uracil, histidine, and glucose. At OD_{600} of 0.1 the cells were washed twice with the same amount of the same medium containing galactose instead of glucose. Four hours of incubation with galactose permitted the expression of the fusion proteins. The cells were then processed as described (35). A mouse monoclonal anti-β-galactosidase antibody (Sigma) and a fluorescein-5-isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment of a sheep anti-mouse IgG antibody (Sigma) were used as primary and secondary antibody, respectively. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI) (Serva).

Plant cells. Protoplasts were collected 9 and 24 hr after transfection and then treated as described (32). The antibodies were the same as for the yeast immunofluorescence experiment. Nuclei were stained with DAPI. Slides were examined with a Zeiss Axiophot microscope.

β-Galactosidase Assay in Plant Cells. Twenty hours after transfection *N. plumbaginifolia* protoplasts were centrifuged and resuspended in 160 μl of extraction buffer (100 mM sodium phosphate, pH 7/10 mM EDTA/0.1% Triton X-100/0.1% *N*-lauroylsarcosine/10 mM 2-mercaptoethanol). The samples were frozen in dry ice and thawed at 37°C three times and centrifuged again. Samples (70 μl) of the supernatants were incubated with 630 μl of *o*-nitrophenyl β-galactopyranoside (4 mg/ml in 0.1 M phosphate buffer, pH 7). At various times the reaction was stopped in 100-μl aliquots by adding 500 μl of 1 M Na_2CO_3 and the A_{420} was measured (36).

RESULTS

Nuclear Targeting in Yeast Cells. We first tested the nuclear localization potential of VirD2 in stably transformed yeast cells. As an antigenic "tag" we chose β-galactosidase, which has been extensively used in nuclear targeting experiments (25, 35, 37–39) because its large size (116 kDa) prevents it from passing through the nuclear pore and it can be fused to other proteins without loss of enzymatic activity (40). To test the two putative NLS regions of VirD2 for nuclear localization activity, three *virD2–lacZ* fusion genes were constructed in the yeast–*E. coli* shuttle vector pMBL214 (29). The fusion plasmids pVD2, pVD2.2, and pVD2.3 express the whole VirD2, VirD2 missing the last 29 amino acids, and VirD2 lacking the first 47 amino acids, respectively. VirD2 and its derivatives were fused at the C terminus to the N terminus of β-galactosidase (see Fig. 2). pVCO expresses β-galactosidase fused to an undecapeptide without expected nuclear targeting properties (see *Materials and Methods* and Fig. 2*b*). The yeast strains containing these plasmids were induced by galactose. The cells were fixed and analyzed by indirect immunofluorescence. The samples were incubated with a monoclonal mouse antibody directed against β-galactosidase and then with FITC-conjugated anti-mouse IgG antibody. Nuclei were stained with DAPI. In the pVD2, pVD2.2, and pVD2.3 samples examined under the microscope, the FITC

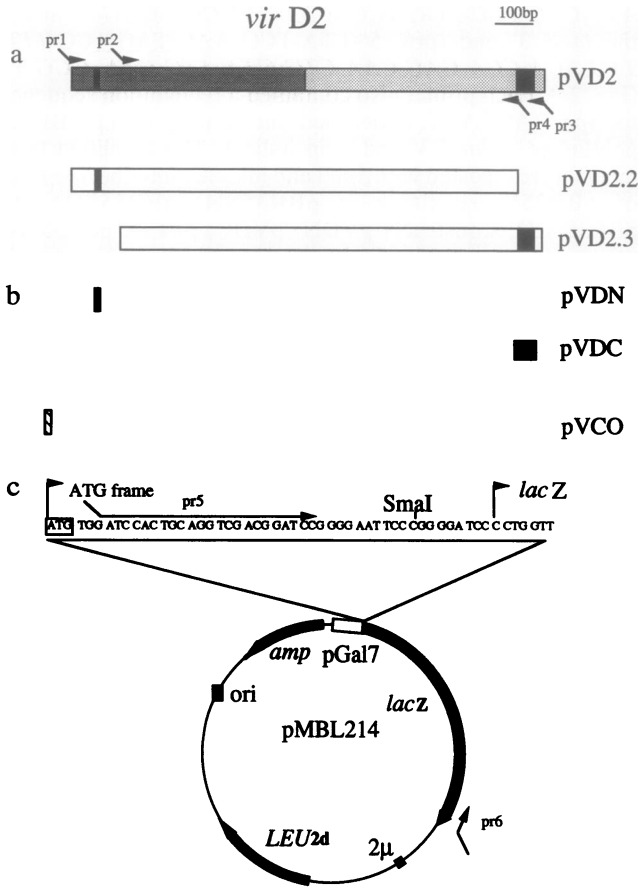

FIG. 2. Construction of *virD2–lacZ* fusions. (*a*) Schematic representation of the constructs encoding the VirD2 protein and N- and C-terminal deletions. Arrows pr1, pr2, pr3, and pr4 indicate the primers used in PCR to fuse the 5′ end of the *lacZ* gene to the entire *virD2* gene (pr1, pr3) to *virD2* lacking its C-terminal NLS (pr1, pr4), and *virD2* lacking its N-terminal NLS (pr2, pr3), resulting in pVD2, pVD2.2, and pVD2.3, respectively. Black bars indicate the NLSs. The dark stippled region in pVD2 corresponds to the highly conserved part of VirD2, whereas the light stippled one corresponds to the less well-conserved part. bp, Base pairs. (*b*) Representation of oligonucleotides fused to *lacZ*. The oligonucleotides encoding the N-terminal NLS (EYLSRKGKLEL) and the C-terminal NLS (PKRPRDRHDGELGGRKRARG) were fused to the 5′ end of *lacZ*, giving pVDN and pVDC. Hatched bar corresponds to a fusion to *lacZ* of the oligonucleotide encoding the N-terminal NLS in the opposite orientation, resulting in pVCO. In this orientation the oligonucleotide encodes a peptide without expected nuclear targeting properties (GVPAFPYGTGT). (*c*) Construction of the *virD2–lacZ* fusion derivatives in the yeast expression plasmid pMBL214. The unique *Sma* I site of pMBL214 was used for the construction of all *lacZ* fusions. Replication origins of yeast (2μ) and *E. coli* (ori) are indicated. *LEU2*d is a yeast selectable marker that is inefficiently expressed and thus allows a high copy number of pMBL214. *amp*, Ampicillin-resistance gene; pGal7, yeast promoter inducible by galactose. Arrows named pr5 and pr6 correspond to the primers used to amplify the *lacZ* derivatives of pVDN, pVDC, and pVCO. The amplified fragments were cloned into the plasmid pBR35S to give pBR35SN, pBR35SC, and pBR35SCO, respectively.

signal was coincident with the nuclear DAPI signal (Table 1). In contrast, in the pVCO sample, FITC stained the entire cell. Thus efficient VirD2-dependent nuclear targeting can be monitored in yeast cells and both the N- and C-terminal regions can function independently to target the β-galactosidase to the nucleus (see Table 1).

To further delimit N-terminal and C-terminal NLSs of VirD2, oligonucleotides coding for 11 amino acids, including the N-terminal NLS, and for 20 amino acids, including the C-terminal NLS, were synthesized and fused to the *lacZ* gene

Table 1. Nuclear targeting of VirD2 and derivatives in yeast and plant cells

	Yeast	Plant
pVD2	N	nt
pVD2.2 Δ(C)	N	nt
pVD2.3 Δ(N)	N	nt
N-terminal NLS	N	N
C-terminal NLS	N	N
Control	C	C

N, nuclear location; C, cytoplasmic location; nt, not tested. Plasmids are described in Fig. 1 and in *Materials and Methods*.

of the pMBL214 vector (plasmids pVDN and pVDC, respectively; Fig. 2*b*). Yeast strains containing either pVDN, pVDC, or pVCO were induced by galactose. The cellular localization of the fusion proteins was again checked by indirect immunofluorescence. Proteins produced by pVDN and pVDC were targeted to the nucleus (as defined by DAPI staining), whereas β-galactosidase produced by pVCO was found in the whole cell (Fig. 3*A* and Table 1). Thus, both the N-terminal and the C-terminal NLS-like sequences of VirD2 are indeed functional nuclear localization signals in yeast.

Nuclear Targeting in Plant Cells. Once the VirD2 NLS sequences were precisely defined in yeast, it was necessary to test them in a plant cell system. The *virD2–lacZ* genes from pVDN, pVDC, and pVCO were amplified by PCR and cloned under the control of the promoter and terminator signals for the CaMV 35S RNA, yielding pBR35SN, pBR35SC, and pBR35SCO, respectively. *N. plumbaginifolia* protoplasts were fixed on microscope slides 9 and 20 hr after transfection with the plasmids and then submitted to indirect immunofluorescence using a mouse anti-β-galactosidase antibody as primary antibody. After incubation with the FITC-conjugated anti-mouse antibody, the FITC staining and the DAPI nucleus-specific staining were examined. About 10% of the transfected cells gave a FITC signal. This signal was clearly located in the nucleus for the constructs pBR35SC and pBR35SN, whereas protoplasts transfected with pBR35SCO gave a diffuse staining of the entire cell (Fig. 3*B*). The untransfected control gave weak staining for all cells. To test whether the diffuse staining observed for pBR35SCO was a consequence of low expression of that particular construct, we measured the β-galactosidase activity of transfected protoplasts. We found the same β-galactosidase activity in a total cell extract of protoplasts transfected with pBR35SCO as in a total extract from protoplasts transfected with pBR35SC, indicating that the diffuse fluorescence observed in cells transfected with pBR35SCO was due to a dilution of the antigen throughout the entire plant cell cytoplasm. We conclude that the two VirD2 NLSs originally defined in yeast are also functional in plant cells.

DISCUSSION

All nuclear proteins and RNAs are transported through the nuclear membrane via an active process controlled by the nuclear pore complex (for review see refs. 13 and 15). This transport requires activation by a NLS belonging either directly to the transported molecule (24, 37) or to an associated helper molecule (41, 42). Because of this tight control, one can imagine that DNA delivered exogenously into the cytoplasm of a eukaryotic cell would not enter the nucleus freely, or at least not efficiently. Indeed, microinjection of DNA into the cytoplasm of mammalian cells has yielded stable transformants at much lower frequency than microinjection directly into the nucleus (43). If we interpret this as inability of the injected DNA to activate the process of nuclear entrance, specialized mechanisms have to be invoked to explain how T-DNA finds its way into the plant nucleus. Our working hypothesis was that the T-DNA requires help

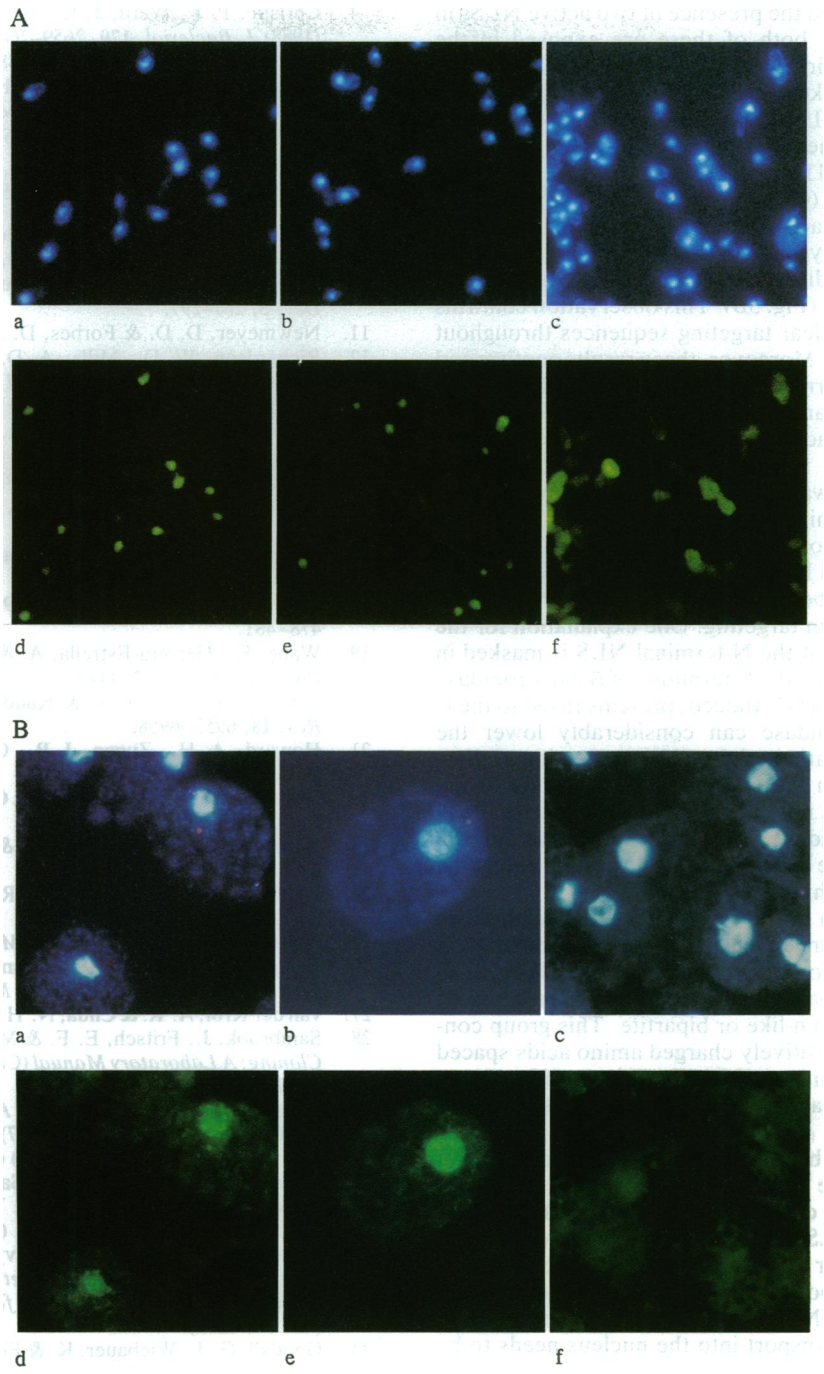

FIG. 3. Analysis by indirect immunofluorescence of intracellular localization of VirD2–β-galactosidase fusion proteins in yeast cells (*A*) and *N. plumbaginifolia* protoplasts (*B*). In both *A* and *B*, *a* and *d* correspond to the N-terminal NLS, *b* and *e* to the C-terminal NLS, and *c* and *f* to the control peptide; *a–c* show DAPI staining of the nucleus; *d–f* show the location of the fusion protein as revealed by FITC fluorescence. (*A*, ×600; *B*, ×900.)

from an attached protein that carries a nuclear localization signal. VirD2 is a good candidate for this function for at least two reasons. (*i*) VirD2 is the only protein that has been found to be tightly (probably covalently) linked to T-DNA (5–8). (*ii*) VirD2 contains two regions of homology with previously described NLSs. One of these putative NLS sequences is located close to the N terminus and the other close to the C terminus of VirD2. In addition, comparison of the amino acid sequences of VirD2 of three different *Agrobacterium* strains revealed that these sequences are particularly well conserved (19). The bipartite C-terminal NLS corresponds to two blocks of conserved amino acids embedded in an otherwise poorly conserved domain of VirD2. Further, *Agrobacterium* mutants expressing a VirD2 protein missing its C-terminal part (including the putative C-terminal NLS) have lost their ability to induce tumors but are efficient in processing T-DNA (5, 19, 20, 44), indicating that this C-terminal part is involved at a later stage of the T-DNA transfer process. The general conservation of the nuclear import process (refs. 24, 25, and 27; P. Wagner and M.N.H., unpublished work) allowed us to perform the initial characterization of the putative VirD2 NLSs in yeast cells. The advantage of yeast as opposed to plants is the ease with which it can be experimentally manipulated. We chose to fuse our constructs to *E. coli* β-galactosidase because it has been shown to act as an efficient antigenic "tag" in yeast (35, 37).

Our analysis confirmed the presence of two active NLSs in VirD2 and proved that both of these are exposed in the tertiary structure of VirD2–β-galactosidase. In yeast, the entire VirD2, VirD2 lacking its N-terminal NLS, and VirD2 lacking its C-terminal NLS are able to target β-galactosidase to the nucleus. We defined two short peptides containing the predicted NLSs, one of 11 amino acids (N-terminal NLS), the other of 20 amino acids (C-terminal NLS), as active nuclear targeting sequences. Each of them is sufficient to target β-galactosidase to the yeast nucleus (Fig. 3*A*). The same sequences were able to direct β-galactosidase to the nuclei of *N. plumbaginifolia* cells (Fig. 3*B*). This observation confirms the conservation of nuclear targeting sequences throughout the eukaryotic kingdom. Moreover, these results confirm and extend the finding of Herrera-Estrella *et al.* (39), who showed that the N-terminal 292 amino acids of VirD2 (70% of VirD2) are able to target β-galactosidase to the plant nucleus. The N-terminal NLS alone was not directly tested by those authors. However, Howard *et al.* (21) tested peptides containing either the N-terminal or the C-terminal NLS of VirD2 fused to the C terminus of *E. coli* β-glucuronidase and found that only the C-terminal NLS has nuclear targeting activity. Our results show that both the C-terminal and N-terminal sequences are efficient in targeting. One explanation for the discrepancy could be that the N-terminal NLS is masked in the fusion protein between the C terminus of β-glucuronidase and the N terminus of VirD2. Indeed, proteins fused to the C terminus of β-glucuronidase can considerably lower the β-glucuronidase enzymatic activity (22), suggesting a physical interaction between the two components of the fusion protein. This problem is not encountered in fusions to the N terminus of β-galactosidase. Another explanation for the discrepancy could be the different methods used to detect the cellular location of the hybrid proteins.

Sequence comparison of the two VirD2 NLSs with those described in the literature reveals that the N-terminal NLS corresponds to the monopartite motif consensus K-R/K-X-R/K (16). The C-terminal NLS belongs to a more complex type, called nucleoplasmin-like or bipartite. This group contains two stretches of positively charged amino acids spaced by about 10 undefined amino acids (17). This motif is present in only 4% of non-nuclear eukaryotic proteins but in 56% of nuclear proteins (18).

We have shown that both nuclear targeting sequences of VirD2 are active in the free protein. However, VirD2 attached to T-DNA might change its conformation so that one or the other (or both) NLS might be masked or more exposed. This is especially true for the N-terminal NLS, since it is very close to a tyrosine proposed to be involved in the covalent linkage of VirD2 to T-DNA (45). Thus the function of these sequences in T-DNA transport into the nucleus needs to be confirmed with a molecule containing an appropriate protein–DNA linkage. Interestingly, a precise deletion of the C-terminal NLS reduces the efficiency of transformation, but does not abolish it completely (Z.K.-N. and L. Rossi, unpublished work). This suggests that the C-terminal NLS is dispensable to a certain extent and indicates that both NLSs might contribute to an efficient transport of T-DNA into the plant nucleus.

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