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Comparison between nuclear localization of nopaline- and octopine-specific *Agrobacterium* VirE2 proteins in plant, yeast and mammalian cells

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SUMMARY

In a unique case of *trans*-kingdom DNA transfer, *Agrobacterium* genetically transforms plants by transferring its DNA segment into the host cell nucleus and integrating it into the plant genome. One of the central players in this process is the bacterial virulence protein, VirE2, which binds the transported DNA molecule and facilitates its nuclear import. Nuclear import of VirE2 proteins encoded by two major *Agrobacterium* strains, nopaline and octopine, has been hypothesized to occur by different mechanisms, i.e. the nopaline VirE2 was imported only into the nuclei of plant cells while the octopine VirE2 also accumulated in the nuclei of animal cells. Here, this notion was tested by a systematic comparison of nuclear import of nopaline- and octopine-specific VirE2 in dicotyledonous and monocotyledonous plants and in living mammalian and yeast cells. These experiments showed that nuclear import of both nopaline and octopine VirE2 proteins is plant-specific, occurring in plant but not in non-plant systems.

INTRODUCTION

Plant genetic transformation by *Agrobacterium* involves the transport of a single-stranded molecule (T-strand) of the transferred DNA (T-DNA) from the bacterial tumour-inducing (Ti) plasmid into the host cell nucleus. Subsequent integration of the T-DNA into the host cell genome results in the formation of crown gall tumours that produce and secrete opines, which are then consumed by *Agrobacterium* (reviewed by Gelvin, 2000; Hooykaas and Beijersbergen, 1994; Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). Agrobacteria are usually classified by a type of opines they specify, the most common strains being octopine- or nopaline-specific (Hooykaas and Beijersbergen, 1994).

Presumably, nuclear import of the T-strand is facilitated by two bacterial virulence (Vir) proteins, VirD2 and VirE2, encoded by the Ti plasmid. VirD2 is an endonuclease, which directly participates in generation of the T-strand. During this reaction, VirD2 becomes covalently attached to the 5′ end of the T-strand molecule (Filichkin and Gelvin, 1993; Ward and Barnes, 1988; Young and Nester, 1988) and then is exported together with the T-strand into the host cell (reviewed by Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). Conversely, VirE2, a single-stranded (ss) DNA binding protein, is most likely exported separately, although its export in association with the T-strand still cannot be ruled out (reviewed by Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). Inside the plant cell, VirE2 combines with the T-strand and VirD2 to form a transfer (T) complex which is then imported into the host cell nucleus with the help of both VirE2 and VirD2 (reviewed by Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000). In addition to nuclear import, both VirD2 and VirE2 may participate in the T-DNA integration process (Mysore *et al.*, 1998; Narasimhulu *et al.*, 1996; Rossi *et al.*, 1996; Tinland *et al.*, 1992).

The prominent role of VirD2 and VirE2 in the T-DNA nuclear import and integration underscore the importance of understanding of the molecular mechanisms by which these proteins are transported into the cell nucleus. To date, it is accepted that VirD2 proteins encoded by both nopaline- and octopine-specific Ti plasmids accumulate in the nucleus by a molecular pathway conserved between plant and animal cells (Guralnick *et al.*, 1996; Rhee *et al.*, 2000; Ziemienowicz *et al.*, 2001; Ziemienowicz *et al.*, 1999). Indeed, VirD2 has been shown to interact with karyopherin α, a nuclear localization signal (NLS) binding protein conserved between plants and animals (Ballas and Citovsky, 1997). In contrast, the details of nuclear import of VirE2 are still controversial. Specifically, nuclear import of the nopaline-type VirE2 occurred in plant (Citovsky *et al.*, 1992, 1994) but not in animal or yeast cells (Guralnick *et al.*, 1996; Rhee *et al.*, 2000) whereas, using *in vitro* nuclear import systems, the octopine-type VirE2 was shown to accumulate in the nuclei of both mammalian and plant cells (Ziemienowicz *et al.*, 1999, 2001). These observations suggest different pathways for nuclear import of the octopine and nopaline VirE2 (and, by implication, the entire T-complexes),

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which is puzzling, because amino acid sequence differences between these proteins are not very significant. Here, we attempted to clear this controversy by a systematic comparison of nuclear import of the nopaline- and octopine-specific VirE2 in dicotyledonous and monocotyledonous plants and in mammalian and yeast cells.

RESULTS

VirE2 is nuclear in plant cells

First, we compared the ability of the nopaline- and octopine-specific VirE2 proteins to localize to the cell nucleus in tissues of

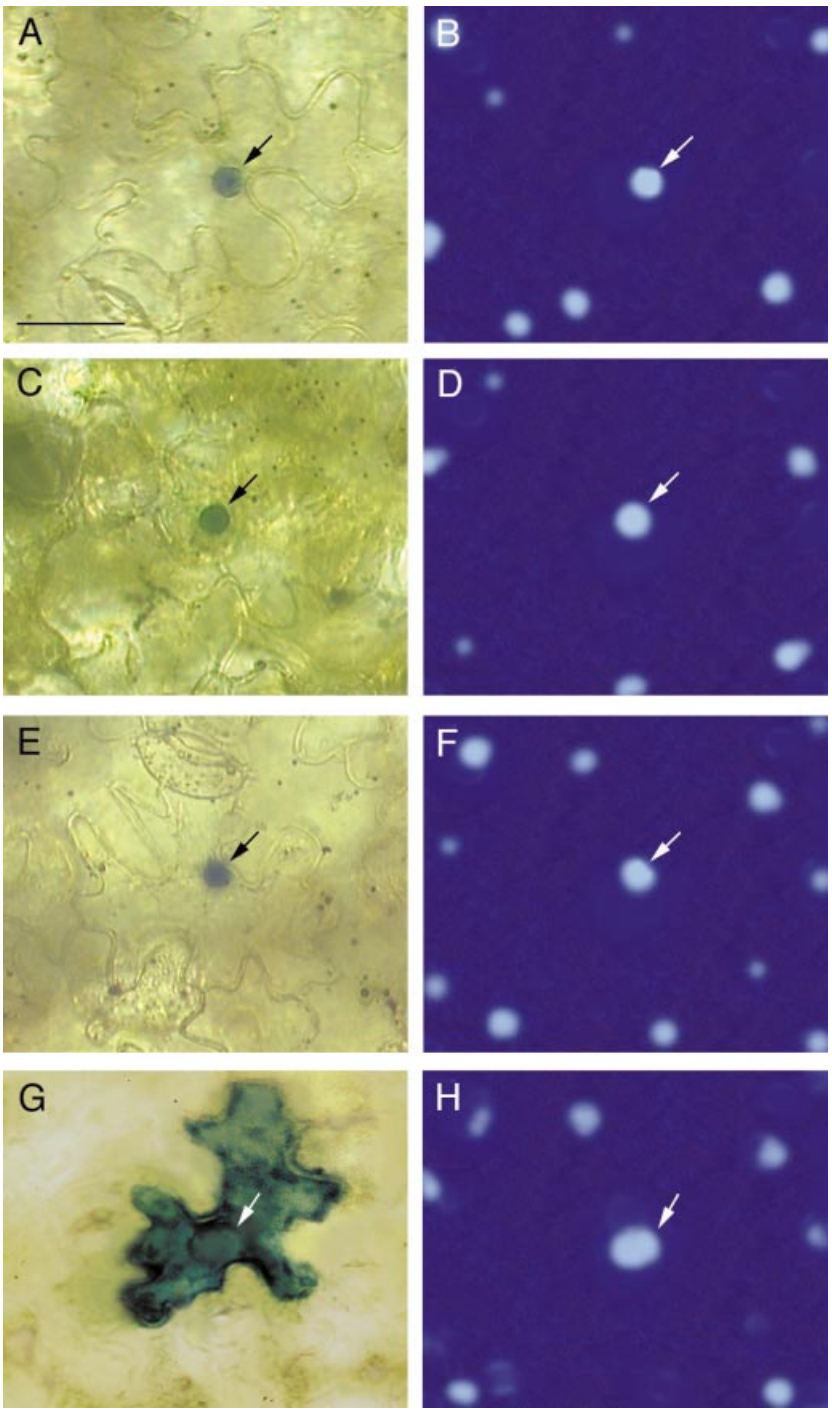

Fig. 1 Nuclear import of GUS-VirE2 in tobacco mesophyll cells. (A, B) Nopaline GUS-VirE2. (C, D) Octopine GUS-VirE2. (E, F) GUS-VirD2. (G, H) free GUS. (A, C, E, G) GUS staining. (B, D, F, H) DAPI staining. Arrows indicate the cell nucleus. Bar = 125 μm.

dicotyledonous (tobacco) and monocotyledonous plants (onion). The proteins were fused to the β-glucuronidase (GUS) reporter and expressed in plant cells following biolistic delivery of the fusion constructs into intact tobacco leaves or living epidermal cells of onion bulb scales. Figure 1 shows that both proteins efficiently accumulated in the tobacco cell nucleus (panels A, C), co-localizing with the nucleus-specific stain, 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 1B,D). In control experiments, GUS-VirD2 also localized to the cell nucleus in tobacco tissues (Fig. 1E,F) whereas free GUS remained cytoplasmic (Fig. 1G,H). Note that, when we used VirE2 fused to GFP instead of the GUS reporter, identical nuclear accumulation patterns were observed (data not shown), further supporting the notion that nuclear import of GUS-VirE2 is due to the VirE2 activity itself.

Essentially the same results were obtained in onion tissues, where the nopaline and octopine GUS-VirE2 accumulated in the cell nucleus. Also, as expected, GUS-VirD2 was nuclear and free GUS stained within the cytoplasm in these cells (Fig. 2). These observations indicate that VirE2 encoded by both nopaline and octopine Ti plasmids is a nuclear protein in dicotyledonous as well as monocotyledonous hosts. Note that onion represents a monocotyledonous host for *Agrobacterium* (Dommisse *et al.,* 1990).

VirE2 remains cytoplasmic in mammalian and yeast cells

To examine whether VirE2 nuclear import is plant-specific or whether it also occurs in non-plant cells, we expressed the nopaline and octopine VirE2 proteins in mammalian and yeast cells. For experiments in mammalian cells, VirE2 was fused to the green fluorescent protein (GFP), introduced into COS-1 cells, and its intracellular localization determined using confocal microscopy. Figure 3A,B shows that neither nopaline nor octopine GFP-VirE2 fusion proteins entered the cell nucleus (dispersed fluorescent signal surrounding fluorescence-free, black nuclei). This lack of nuclear import was specific for VirE2 because GFP-VirD2 efficiently accumulated in the COS-1 cell nuclei (Fig. 3C), indicating that these cells are competent for nuclear import. Note that the confocal optical sections, shown in Fig. 3, with the plane of focus through the cell nucleus detect intranuclear accumulation of the GFP label rather than its perinuclear binding.

Next, the nopaline and octopine VirE2 were examined for their ability to enter the yeast cell nucleus using a genetic assay for functional nuclear import (Rhee *et al.*, 2000). In this approach, a gene encoding the bacterial LexA protein was modified (mLexA), to abolish its intrinsic nuclear targeting activity, and fused with a sequence coding for the activation domain of the yeast Gal4p (Gal4AD). If a protein of interest fused to mLexA-Gal4AD (nuclear import assay hybrid, NIA) enters the yeast cell nucleus, it activates the expression of the reporter *HIS3* gene, resulting in cell growth on a histidine-deficient medium (Rhee *et al.*, 2000).

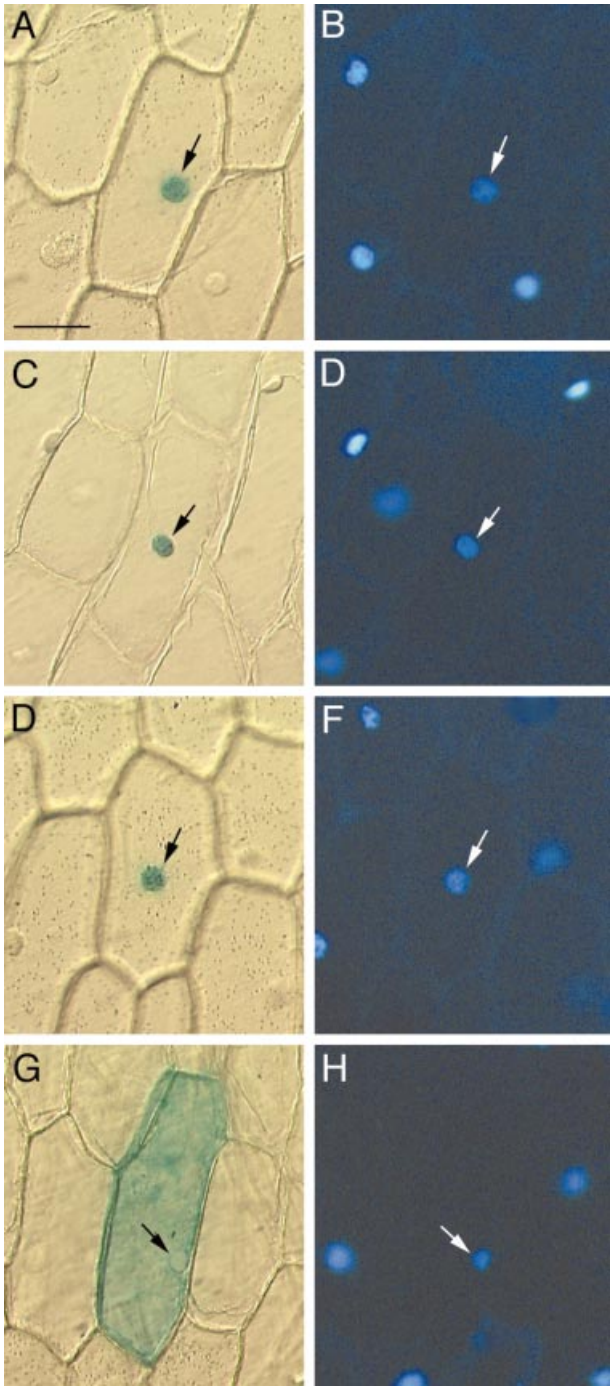

Fig. 2 Nuclear import of GUS-VirE2 in onion epidermal cells. (A, B) Nopaline GUS-VirE2. (C, D) Octopine GUS-VirE2. (E, F) GUS-VirD2. (G, H) free GUS. (A, C, E, G) GUS staining. (B, D, F, H) DAPI staining. Arrows indicate the cell nucleus. Bar = 100 μm.

Figure 4A shows that NIA fusions with the nopaline and octopine VirE2 did not promote cell growth (lanes 2 and 3), indicating the lack of nuclear uptake. Expression of NIA-VirD2, on the other hand, induced cell growth (lane 4), indicating nuclear

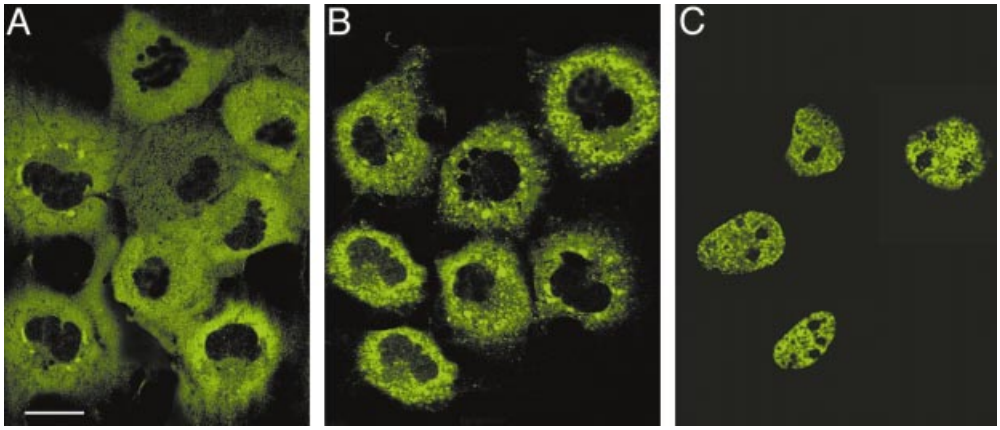

Fig. 3 Expression of GFP-VirE2 in COS-1 cells. (A) Nopaline GFP-VirE2. (B) Octopine GFP-VirE2. Dispersed fluorescence surrounding the signal-free, black cell nucleus represents the cytoplasmic localization of GFP-VirE2. (C) GFP-VirD2. The fluorescent signal is concentrated exclusively in the cell nucleus. Bar = 20 μm.

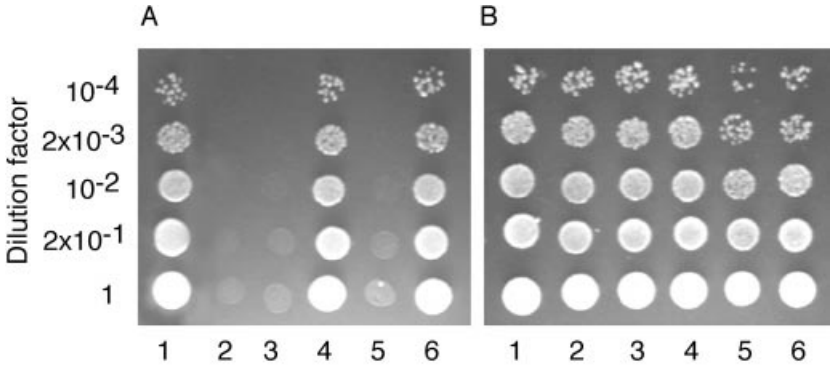

Fig. 4 Functional nuclear import assay in yeast cells. Yeast cells expressing the indicated proteins were grown under (A) selective nuclear import conditions, i.e. histidine and tryptophan double-dropout medium supplemented with 5 mM of 3-amino-1,2,4-triazole (3AT), or (B) nonselective nuclear import conditions, i.e. tryptophan single-dropout medium. 1, nopaline NEA-VirE2; 2, nopaline NIA-VirE2; 3, octopine NIA-VirE2; 3, NIA-VirD2; 5, NIA-2DVir; 6, octopine NEA-VirE2.

import (Fig. 4A). NIA-VirD2 nuclear import was due to the presence of the VirD2 sequence because when the latter was fused to NIA in reverse orientation, producing the NIA-2DVir hybrid, no cell growth was observed (Fig. 4A, lane 5). To demonstrate directly that the absence of cell growth following expression of NIA-VirE2 fusions was due the lack of their nuclear import, we used VirE2 fusions with a reporter, termed NEA, carrying the SV40 large T-antigen NLS between the mLexA and Gal4AD sequences (Rhee *et al.*, 2000). Figure 4A shows that both nopaline and octopine NEA-VirE2 fusions were imported into the yeast cell nucleus, as indicated by the efficient cell growth (lanes 1 and 6, respectively). In control experiments, cells harbouring all NIA fusions grew well in the presence of histidine (Fig. 4B), indicating that neither of the protein hybrids adversely and nonspecifically affected yeast cell physiology.

DISCUSSION

T-DNA transport into the host cell nucleus is the central event in genetic transformation of plants by *Agrobacterium*. The bacterial protein VirE2, one of the major players in this process, cooperatively binds the transported DNA molecule, shapes it into a transferable form, and, together with VirD2, mediates its nuclear uptake (reviewed by Sheng and Citovsky, 1996; Tzfira and Citovsky, 2000; Tzfira *et al.*, 2000; Zupan *et al.*, 2000); furthermore, the nopaline-typeVirE2 alone may actively import ssDNA into the plant cell nucleus when microinjected into living plant cells (Zupan *et al.*, 1996). Previously, the nopaline-type VirE2 was shown to accumulate in the nucleus of plant but not of animal cells (Citovsky *et al.*, 1992, 1994; Guralnick *et al.*, 1996; Rhee *et al.*, 2000). On the other hand, the octopine-type VirE2 has been shown to enter the animal cell nuclei in an *in vitro* system (Ziemienowicz *et al.*, 1999). Thus, the nopaline and octopine VirE2 proteins may differ in their ability to function in non-plant systems, suggesting different pathways for nuclear import of T-DNA derived from the octopine and nopaline Ti plasmids. Here, we argue against this hypothesis, demonstrating that, when studied *in vivo*, nuclear import of the nopaline and octopine VirE2 proteins occurs in plant but not in mammalian or yeast cells. These results also suggest that the nuclear import of VirE2 in a cell-free system (Ziemienowicz *et al.*, 1999) may differ from that within living cells. This difference between living cells and *in vitro* nuclear import systems may also explain the recently published observation that octopine VirE2 alone does not mediate the import of ssDNA into the nuclei of

permeabilized plant protoplasts (Ziemienowicz *et al.*, 2001). The lack of nuclear import of VirE2 in animal and yeast cells implies an involvement of plant-specific host cellular factors that interact with VirE2 and facilitate its nuclear uptake. It is important to note, however, that during the recently reported *Agrobacterium* infection of human cultured cells (Kunik *et al.*, 2001), the VirE2 nuclear import function may be substituted by as an yet unknown cellular protein(s).

EXPERIMENTAL PROCEDURES

Plasmids

VirE2 and VirD2 sequences were PCR amplified and fused as *Bam*HI-*Xho*I fragments to the GFP reporter gene in pEGFP-C1 (Clontech) for expression in mammalian cells, to the GUS reporter gene in pRTL2-GUS (Restrepo *et al.*, 1990) for expression in plant tissues, or to the NIA or NEA reporters of pNIA or pNEA (Rhee *et al.*, 2000) for expression in yeast. For expression of VirD2 in reverse orientation, it was PCR amplified as a *Xho*I-*Bam*HI fragment and subcloned into pNIA. Free GUS and GUS-VirD2 were expressed from pRTL2-GUS (Restrepo *et al.*, 1990) and pGD plasmids (Howard *et al.*, 1992), respectively. All PCR reactions were performed using a high fidelity Pfu DNA polymerase (Promega) and their products were verified by nucleotide sequencing.

Nuclear import in plant cells

For expression in plant tissues, 1 μg DNA adsorbed on to 0.5 mg of 1 μm gold particles according to the instructions of the manufacturer (Bio-Rad, CA) were microbombarded into the leaf mesophyll of greenhouse-grown *Nicotiana tabacum* plants or into the epidermis of onion bulb scales as described (Huang and Zhang, 1999) at a pressure of 150 psi using a portable Helios gene gun system (Model PDS-1000/He, Bio-Rad, CA). After incubation for 16 h at 25 °C to allow expression of the transfected DNA, the leaf disks were stained for GUS activity for 3 h (Nam *et al.*, 1999) and observed under a Zeiss Axiophot microscope. DAPI staining was used to verify the location of the cell nucleus.

Nuclear import in mammalian and yeast cells

For nuclear import in mammalian cells, 2.5×10^4 cells/cm^2 of COS-1 cells were transformed with the expression constructs using FuGENE 6 (Roche) according to the manufacturer's instructions, cultured in DMEM medium supplemented with 10% bovine calf serum for 24 h at 37 °C, and viewed under a Zeiss LSM 410 laser scanning confocal microscope equipped with a 485 nm excitation argon laser and a 527 nm GFP emission filter. Yeast nuclear import was assayed in L40 yeast cells (Hollenberg *et al.*, 1995) as described (Rhee *et al.*, 2000).

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