Research Article

The significance of C-terminal NLS sequences of VirD2 in its nuclear localization in *Saccharomyces cerevisiae*

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Abstract

Agrobacterium tumefaciens is capable of gene transfer to both plant and non-plant organisms. Indeed, upon infection of eukaryotic cells, *Agrobacterium tumefaciens* transfers a piece of its tumor inducing (Ti)-plasmid, called T-DNA, to the host cell nucleus, which subsequently integrates into the host genome. The VirD2 virulence protein which has relaxase endonuclease activities covalently binds to the 5'end of T-DNA and facilitates its transfer, nuclear localization and integration into the host genome in collaboration with the interacting proteins of the host cell. The VirD2 is essential for *Agrobacterium*–mediated transformation of both plants and non-plant cells. Here, using yeast Green Flourescent Protein (yGFP) technology, we studied the subcellular localization of VirD2, expressed in the model eukaryote *Saccharomyces cerevisiae*. Fluorescence microscopy showed that an N-terminal yGFP fusion of VirD2 (i.e. 5' GFP-VirD2 3'), was located in the nucleus of yeast. With C-terminal fusions of VirD2 to yGFP (i.e. 5' VirD2-GFP 3'), no particular subcellular concentration of fluorescence was seen. This further confirms nuclear localization of VirD2 in eukaryotic cells and more importantly highlights the role of Nuclear Localization Signal sequences (NLS) of the C-terminal of VirD2 in this phenomenon.

***Keywords*:** VirD2, Nuclear delivery, GFP, *Agrobacterium, Saccharomyces cerevisiae*

Introduction

Nuclear gene delivery possesses great potential for its use in basic science, biotechnology, agriculture, and medicine. Developing gene transfer technologies has become one of the most intensively investigated strategies for current basic and clinical research. *Agrobacterium tumefaciens* is a Gram-negative phytopathogen which is able to transfer and integrate up to 150 kb single-stranded DNA (ssDNA) into the infected cell's cytoplasmic and nuclear genome (Soltani et al., 2008). This ability is mainly due to the presence of a tumor inducing (Ti) plasmid in *Agrobacterium*. The Ti plasmid encodes a number of virulence proteins (Vir) that mediate the formation of a single stranded DNA copy (T-strand) of a part of the Ti-plasmid and transferring of it across the kingdom barriers to integrate into the host genome (Citovsky et al., 2006). Upon induction of the virA-virG two-component regulatory system, the virulence (vir) regulon expresses several Vir effector proteins which play different roles in the tumor induction process. Among those, the VirD2 relaxase together with VirD1 and VirC1 is responsible for the formation of the T-strand. The VirD2 virulence protein covalently bounds to the liberated 5′ phosphate of the T- strand and through combined action of three NTP-binding/hydrolyzing proteins VirB4, VirB11, and VirD4 translocates, as a pilot protein, the T-strand to the recipient cell via a Type IV Secretion System. Inside the host cell around 600 VirE2 proteins cover the T-single strand. Both VirD2 and independently transferred nuclear localization signal sequence (NLS) containing VirE2 facilitate the import of the T-complex into the host nucleus. VirD2 has two nuclear localization sequences (NLS), one located in its N-terminal region and the second bipartite NLS sequence located in the C-terminal region (Wang et al., 1990; Howard et al., 1992). It has been shown that both VirD2 and VirE2 proteins have interaction with plant importn proteins implicating host factors in the nuclear entry of T-complex (Ballas and citovsky, 1997; Tzfira et al., 2001; Li et al., 2005).

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Furthermore, VirD2 interacts with a number of plant cyclophilins, a conserved cyclin-dependent kinase-activating kinase (Cak2M), and the TATA-binding protein (TBP) (Bako et al., 2003). Inside the host nucleus, VirD2 relaxase may influence the integration of T-DNA into the genome, although this is largely mediated by host factors (van Attikum et al., 2001; 2003). The unique mechanism by which *Agrobacterium* translocates any ssDNA molecule offers novel possibilities for gene transfer into fungal and mammalian cells (Soltani et al., 2008; Haghighi et al., 2013). Here, we studied the subcellular localization of the pilot protein VirD2 in yeast cell as a model eukaryote and the role of C- and N-terminal of NLS in this event.

Materials and Methods

Strains and media

E. coli strain XL1-blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac−F' [proAB+ lacIq lacZΔM15 Tn10*] Tc^r) was used for all cloning processes (Stratagene). *E. coli* was grown at 37°C in Luria-Bertani (LB) or TB medium containing either 100µg/ml ampicillin or 60 µg/ml kanamycin. S. cerevisiae strain CEN.pk113-3B (*MATα his3Δ1 ura3-52*) was used for the green fluorescent protein (GFP) localization studies. All yeast strains were grown at 30°C in either YPD or MY supplemented with appropriate nutrients, i.e. 20 µg/ml adenine, 30µg/ml histidine, 20 µg/ml leucine, 30µg/ml lysine, and 20 µg/ml tryptophan (Sherman, 1991; Zonneveld, 1986).

Nucleic acid manipulations

All nucleic acid manipulations for plasmid construction were performed by standard protocols (Sambrook et al., 1989). For plasmid DNA isolation from *E. coli*, the QIAprep mini spin kit (Qiagen) was used. For isolation of plasmid from the yeast cells the same kit was used, after adding lyticase (1 mg/ml) to buffer P1. Isolated plasmids from yeast were amplified in *E. coli* XL1-blue.

Plasmid constructions

For the construction of pGBDKc1-virD2 the 3'-end of the virD2 open reading frame, lacking the first 379 bp, was obtained by PCR on plasmid pVD43 (Rossi et al, 1993). After digestion with *Sal*I and *Bgl*II, this part was cloned into pGBDK-C1 (van Hemert et al, 2003) digested with the same enzymes. The 5'-end of the virD2 open reading frame (379 bp) was obtained by PCR on plasmid pVD43 (Rossi et al, 1993) using the primers VirD2SalIp2 (5'-ACGCGTCGACGTCATGCCCCGATCGCGCTC AAG-3'), introducing a *Sal*I restriction site upstream of the ATG start codon, and VirD2p2 (5'-TATTCGGTCCTTCCTGTCTCTAGGTCCCCCC-3'). Subsequently, this fragment was digested with *Sal*I and introduced into the *Sal*I site of pGBDK containing the 3'-end of VirD2.

To make fusions between VirD2 and yeast enhanced GFP, an *Xma*I-*Eco*RI fragment with virD2 obtained from pGBDKc1-virD2 plasmid was cloned into the *Xma*I-*Eco*RI restriction sites of pUG34, pUG35 and pUG36 GFP-vectors (U. Güldener and J. H. Hegemann, unpublished data). In pUG34-virD2 and pUG36-virD2 vectors, virD2 is tagged with GFP at its N-terminus, and in pUG35-virD2 vector, virD2 is tagged with GFP at its C-terminus, and expressed under control of the *MET17* (*alias MET25*) promoter. Plasmids are listed in Table 1. New constructs were confirmed by both restriction analyses and DNA sequencing (BaseClear, The Netherlands). The GAD-fw primer 5'-GATGAGAAGATACCCCACC-3' was used for sequencing of the samples of the genomic libraries.

Table 1. Plasmids used in this study.

Plasmid	Features
pGBDKc1.*vir*D2 (pRUL1131)	*ADH1* promoter, Gal4 BD, AmpR, *TRP1*, Kan, ori, carrying *vir*D2
pUG34	*MET25* promoter, *HIS3*, CEN6/ARS4, AmpR, ori, N-terminal yGFP fusion site
pUG34-*vir*D2 (pRUL1146)	Expresses N-terminal yGFP fusion to VirD2
pUG35	*MET25* promoter, *URA3*, CEN6/ARS4, AmpR, ori, C-terminal yGFP fusion site
pUG35-*vir*D2 (pRUL1147)	Expresses C-terminal yGFP fusion to VirD2
pUG36	*MET25* promoter, *URA3*, CEN6/ARS4, AmpR, ori, N-terminal yGFP fusion site
pUG36-*vir*D2 (pRUL1148)	Expresses N-terminal yGFP fusion to VirD2

Transformation protocols

E. coli XL1-blue was transformed using regular heat shock protocol (Takahashi et al., 1992). For transformation of *S. cerevisiae* strains lithium acetate protocol was carried out (Gietz and Woods,

2002) and transformants were selected on MY medium supplemented with appropriate nutrients (Zonneveld, 1986).

Microscopy

For 4', 6-Diamidino-2-phenylindole (DAPI) staining of nuclei, overnight cultures from yeast strain CEN.pk113-3B containing GFP-fused VirD2 were harvested by centrifugation and resuspended in 1 ml of 70% ethanol (Hašek and Streiblová, 1996). After 5 min, the cells were again harvested and resuspended in 25 µL of 0.1 µg/ml DAPI. 5 µL of DAPI-stained yeast suspensions were then used for microscopy. Accordingly, 5 µL of overnight cultures were taken for fluorescence microscopy with a Zeiss Axio-plan-2 imaging microscope. GFP was excited at 488 nm, and emission was detected at 514-564 nm.

Results

Plasmid constructions

To confirm the virD2 insertion in GFP-containing plasmids, the restriction enzyme analyses were followed by sequencing of the constructs. *Sma*I, *Eco*RI, *Eco*RV restrictions resulted in the expected bands for each construct (Figure 1). The constructs (pUG34-virD2, pUG35-virD2, and pUG36-virD2) were checked further by sequencing (Data not shown).

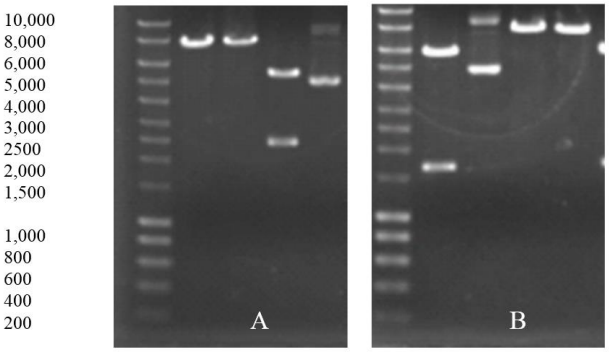

Figure 1. Representative restriction alanylses of virD2-gfp plasmid constructs. A, pUG35.virD2 restricted with EcorRI (ca. 7550 bp), SmaI (ca. 7550 bp), EcoRV (ca. 5132 bp & 2418 bp) & control respectively. B, pUG36.virD2 restricted with *Eco*RV (ca. 5852 bp & 1692 bp), control, *Eco*RI (ca. 7550 bp), *Sma*I (ca. 7550 bp), respectively. The numbers indicate the sizes of DNA ladder bands (bp).

To determine the localization of VirD2 in S. cerevisiae we expressed N- and C-terminal fusions of this protein with yGFP in this organism. Fluorescence microscopical analysis of yeast transformants was performed on overnight grown cells. Cells expressing an N-terminal fusion of VirD2 with yGFP (from both pUG34 and pUG36) revealed a typical nuclear localization of this protein (Figure. 1. A & D). DAPI staining of these cells confirmed the nuclear localization (Figure. 1. B & E). We were unable to detect nuclear localization of yGFP fluorescence in yeast cells expressing a C-terminal fusion of VirD2 with yGFP (Data not shown).

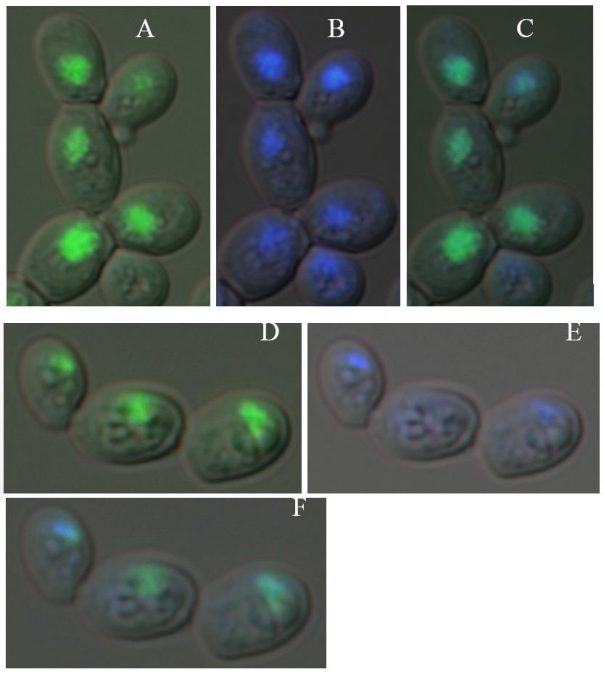

Figure 2. Subcellular localization of VirD2 fused at its N-terminus to C-terminus of GFP from both pUG34 (A-C) and pUG36 (D-F) in S. cerevisaie strain CEN.pk113-3B. A & D, fluorescence microscopy of expressed GFP proteins. B & E, DAPI staining of the same cells. C & F, Superimposition of figures A/B and D/E. Cells are visualized by a Zeiss Axio-plan-2 imaging microscope.

Discussion

Currently, gene transfer is limited largely by the fact that the issue of nucleic acid delivery has not been adequately resolved (Anderson, 1998). The nature's genetic engineer *Agrobacterium tumefaciens* translocates any ssDNA molecule to both eukaryotic and prokaryotic cells (Soltani et al., 2008). This offers novel possibilities for gene transfer into any eukaryotic cells. For a better understanding of the function of VirD2 in eukaryotic cells, the genetic model organism S. cerevisiae was recruited as a host to analyze the subcellular localization of VirD2 expressed in its cells.

The T- DNA of *Agrobacterium* transferred to the host cell needs to translocate to the nucleus to integrate in the genome. Inside the cytoplasm of the

host cell the T-DNA which is bound to VirD2 most likely is covered by VirE2 proteins. Both VirD2 and VirE2 have nuclear localization signals (NLS) sequences which mediate the import of the whole T-complex into the nucleus of plant cells (Citovsky et al., 1992; Tinland et al., 1992). We hypothesized that this might also happen in other eukaryotic cells as *S. cerevisiae*. C-terminal and N-terminal fusions of the virD2 to yGFP were expressed in yeast cells to visualize the subcellular localizations of them. As it is shown in Figure 1, VirD2 fused at its N-terminus to yGFP localized almost exclusively to the nuclei of *S. cerevisiae*. Nuclear localization of VirD2 in yeast cells is consistent with its localization in plant and mammalian cells (Citovsky et al., 1992; Tinland et al., 1992; Relić et al., 1998; Ziemienowicz et al., 1999; Ziemienowicz et al., 2001). However, with C-terminal fusions of VirD2 to GFP, fluorescence was largely uniform throughout the cells without nuclear concentration. VirD2 has an NLS sequence located in its N-terminal region and a bipartite NLS sequence located in the C-terminal region (Wang et al., 1990; Howard et al., 1992). In plants, it has been shown that N-terminal sequences of VirD2, containing 70% of the protein, could target β-galactosidase to the nucleus (Herrera-Estrella et al., 1990). It has also been shown that both the C- and N-terminal sequences of VirD2, when fused at their C-terminus to β-galactosidase, were able to direct β-galactosidase to the nuclei of plant cells (Tinland et al., 1992). Also, either the N- or C-terminus of VirD2 was sufficient to target the GFP fused protein to the nucleus of mammalian cells (Relić et al., 1998). In contrast, import of DNA into the nucleus of mammalian cells by VirD2 is dependent on the C-terminal NLS of VirD2 (Ziemienowicz et al., 1999; 2001). Similarly only the C-terminal NLS of VirD2, not the N-terminal NLS, fused to the C-terminus of β-glucoronidase targets the recombinant protein to the plant nuclei (Howard et al., 1992). The discrepancy between those and our observations could be due to the effect on NLS function of different reporter genes fused N- or C-terminally to VirD2, the different cells used for localization studies and different lengths of virD2 used. Our observation indicates the significance of C-terminal NLS in VirD2 localization in yeast nuclei. C-terminal fusions of VirD2 to GFP may block the function of the NLS, but when GFP is fused to the N-terminal region of VirD2 the NLS at the C-terminus is still functional and mediates the nuclear localization of the recombinant protein. Overall, in accordance to its function in plant and mammalian cells, VirD2 moves to the nucleus of its fungal host cells, and this function is mainly based on its bipartite NLS sequence located in the C-terminal region of the protein.

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