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Host genes involved in Agrobacterium-mediated transformation

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Chapter 5

Expression of Agrobacterium VirD2 in Saccharomyces cerevisiae: subcellular localization and interaction partners

Abstract

Upon infection of eukaryotic cells, *Agrobacterium tumefaciens* transfers a piece of its tumor inducing plasmid, T-DNA, to the host cell. The VirD2 virulence protein which is covalently bound to the T-DNA facilitates its transfer, nuclear localization and possibly integration into the host genome in collaboration with the interacting proteins of the host cell. VirD2 is essential for *Agrobacterium*-mediated transformation of both plants and yeast cells. Here, we studied the subcellular localization of VirD2 expressed in yeast cells and started with the identification of yeast proteins interacting with VirD2 using the yeast two-hybrid system. Fluorescence microscopy showed that an N-terminal Green Fluorescent Protein (GFP) fusion of VirD2 is located in the nucleus of yeast. Using yeast two hybrid screening of *S. cerevisiae* genomic DNA libraries to identify the proteins interacting with VirD2, we identified three prey plasmids containing: *AGC1*, encoding a mitochondrial amino acid transporter, *CDC55*, encoding a non-essential regulatory subunit B of protein phosphatase 2A, and *YMR317W*, an open reading frame of unknown function. In addition, sixteen other prey plasmids were isolated that repeatedly activated the reporter gene in the presence of the VirD2 bait plasmid, but do not have in-frame fusions. Among these, DNA inserts with *HAP4*, *MIG2* and parts of an rDNA region on the right arm of chromosome XII (containing rDNA18, rDNA25, rDNA37, rDNA58, the non-coding regions between them *ITS1*, *ITS2*, *NTS2* and rDNA *ARS1200-1*) were identified 2, 4 and 5 times, respectively.

Introduction

Agrobacterium tumefaciens is a soilborn plant pathogen, that genetically transforms wounded plant cells to produce opines which are exclusively used by agrobacteria in the rhizosphere. 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 (for review see: Citovsky *et al.*, 2006). There, the genes located on the T-DNA encode proteins that modify the host cell to produce plant hormones and

tumor specific metabolites called opines (for review of genes see Zhu *et al.*, 2000). Upon induction of the *virA-virG* regulatory system by phenolics released from wounded plant cells, the virulence (*vir*) regulon expresses several Vir 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 which is covalently bound to the liberated 5' phosphate of the T-strand translocates the T-strand to the recipient cell via a Type IV Secretion System that is formed by VirB1-11/VirD4 proteins. Inside the host cell VirE2 proteins cover the T- strand. Both VirD2 and VirE2 have nuclear localization sequences which may facilitate the import of the T-complex into the host nucleus. It has been shown that both proteins have interaction with plant importins implicating host factors in the nuclear entry of T-complex (Ballas and Citovsky, 1997; Tzfira *et al.*, 2001, 2004; Li *et al.*, 2005). Furthermore, VirD2 interacts with a number of host cyclophilins, a conserved cyclin-dependent kinase-activating kinase (Cak2M), and the TATA-binding protein (TBP) (Bako *et al.*, 2003). Inside the host nucleus, VirD2 may influence the integration of T-DNA into the genome, although this is largely mediated by host factors (van Attikum *et al.*, 2001; 2003).

Under laboratory conditions, induced *Agrobacterium* transfers T-DNA not only to plants, but also to algae, yeasts, filamentous fungi, and mammalian cells (Soltani *et al.*, 2008). In parallel to *Arabidopsis*, the yeast *Saccharomyces cerevisiae* has become a model in our group to investigate the host effectors of the *Agrobacterium*-mediated transformation process (Bundock, 1999; van Attikum, 2003; This thesis). Through the use of this organism it was shown that the integration of T-DNA into the host genome is mediated by the host DNA repair machinery via either homologous recombination or non-homologous end-joining (van Attikum *et al.*, 2001; 2003). Moreover, in a forward genetic screening assay a large number of yeast genes were identified whose deletion significantly decreased or increased the outcome of the *Agrobacterium*-mediated transformation efficiency (Chapter 3 and 4).

The yeast two-hybrid system has enabled the interaction studies of *Agrobacterium* Vir proteins with *Arabidopsis thaliana* gene expression libraries (Ballas and Citovsky, 1997; Bako *et al.*, 2003; Deng *et al.*, 1998; García-Rodríguez *et al.*, 2006; Hwang and Gelvin, 2004; Schrammeijer *et al.*, 2001). However, yeast binding partners of

virulence proteins have not yet been investigated. Using yeast two hybrid screening of *S. cerevisiae* genomic DNA libraries to identify proteins interacting with VirD2 we identified three prey plasmids containing: *AGC1*, *CDC55*, and *YMR317W*. In addition, sixteen other prey plasmids were isolated that repeatedly activated the reporter gene in the presence of the VirD2 bait plasmid, but do not have in-frame fusions. We also studied the subcellular localization of VirD2 in yeast cells and we found that an N-terminal GFP fusion of VirD2 localized to the nucleus of yeast.

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 60µg/ml kanamycin. *S. cerevisiae* strain HF7c (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4-542, gal80-538, LYS2::GALI UAS-GAL1TATA-HIS3, URA3::GAL4 17mers(x3)-CyC1TATA-lacZ*) was used as the reporter strain in yeast two-hybrid screenings (Feilotter *et al.*, 1994). *S. cerevisiae* strain CEN.pk113-3B (*MATa 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. 20µg/ml adenine, 30µg/ml histidine, 20µg/ml leucine, 30µg/ml lysine, and 20µg/ml tryptophan (Sherman, 1991; Zonneveld, 1986).

Nucleic acid manipulations

All nucleic acid manipulations 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 lyticase (1 mg/ml) was added 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 *SalI* and *BglII*, 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 VirD2*SalI*p2 (5'- ACGCGTCGACGTCATGCCCCGATCGCGCTCAAG - 3'), introducing a *SalI* restriction site upstream of the ATG start codon, and VirD2p2 (5'-TATTCGGTCCTTCCTGTCTCTAGGTCCCCC- 3'). Subsequently, this fragment was digested with *SalI* and introduced into the *SalI* site of pGBDK containing the 3'-end of VirD2.

To make fusions between VirD2 and yeast enhanced GFP, an *XmaI*-*EcoRI* fragment with *virD2* obtained from pGBDKc1-*virD2* plasmid was cloned into the *XmaI*-*EcoRI* 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 sequences of the primers used for sequencing of the samples of the genomic libraries and for the sequencing of pMVHis.*virD2* are shown in Table 2.

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 μ L of 0.1 μ g/ml DAPI. 5 μ L of DAPI-stained yeast suspensions were then used for microscopy. Accordingly, 5 μ L 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.

Table 1. Plasmids

Plasmid	Features	Reference
<i>S.cerevisiae</i> DNA library in pGADc1	Gal4 AD fused to genomic library, AmpR, <i>LEU2</i> , ori,	James <i>et al.</i> , 1996
<i>S.cerevisiae</i> DNA library in pGADc2	Gal4 AD fused to genomic library, AmpR, <i>LEU2</i> , ori,	James <i>et al.</i> , 1996
<i>S.cerevisiae</i> DNA library in pGADc3	Gal4 AD fused to genomic library, AmpR, <i>LEU2</i> , ori,	James <i>et al.</i> , 1996
pGBDKc1.virD2 (pRUL1131)	<i>ADHI</i> promoter, Gal4 BD, AmpR, <i>TRP1</i> , Kan, ori, carrying <i>virD2</i>	A.Briancon-Marjollet and H. van Attikum (unpublished data)
pUG34	<i>MET25</i> promoter, <i>HIS3</i> , CEN6/ARS4, AmpR, ori, N-terminal GFP fusion site	U. Güldener and J. H. Hegemann, (unpublished data)
pUG34- <i>virD2</i> (pRUL1146)	Expresses N-terminal GFP fusion to VirD2	This study
pUG35	<i>MET25</i> promoter, <i>URA3</i> , CEN6/ARS4, AmpR, ori, C-terminal GFP fusion site	U. Güldener and J. H. Hegemann, (unpublished data)
pUG35- <i>virD2</i> (pRUL1147)	Expresses C-terminal GFP fusion to VirD2	This study
pUG36	<i>MET25</i> promoter, <i>URA3</i> , CEN6/ARS4, AmpR, ori, N-terminal GFP fusion site	U. Güldener and J. H. Hegemann, (unpublished data)
pUG36- <i>virD2</i> (pRUL1148)	Expresses N-terminal GFP fusion to VirD2	This study
pMVHis (pRUL181)	Gal1 promoter, <i>MAL32</i> , AmpR, Ura3, ori, 6His tag	M. van Hemert, 2003
pMVHis- <i>virD2</i> (pRUL1150)	6 $\times$ His tag fused with VirD2 in pMVHis	This study

Table 2. Nucleotide sequence of primers used.

Name	Sequence 5' - 3'	Function
GAD-fw	GATGAGAAGATACCCACC	Genomic library sample sequencing
pYES-1	CGTGAATGTAAGCGTGAC	pMVHis- <i>virD2</i> sequencing (forward primer)
pYES-2	GCAGCTGTAATACGACTC	pMVHis- <i>virD2</i> sequencing (reverse primer)

Yeast Two-Hybrid screening

pGBDKc1.virD2 expressing a fusion between the Gal4 DNA binding domain and VirD2 was used as a bait plasmid in the yeast two hybrid screens using the yeast reporter strain HF7c. The yeast strain carrying this plasmid was transformed with 50 µg of each of the C1, C2 and C3 yeast genomic libraries (James *et al.*, 1996). Activation of *HIS3* reporter was selected on MY medium containing adenine and lysine. The total number of transformants was determined on MY medium containing adenine, lysine and histidine. Histidine positive colonies were further investigated by β-galactosidase activity in a filter assay. For this, yeast colonies were placed on a filter paper soaked in 1 mg/ml X-gal, 50 mM phosphate buffer pH 7.6, 1 mM MgCl₂, and 2 mM 2-mercaptoethanol. Subsequently, filters were immersed in liquid nitrogen and placed on three layers of filter papers soaked in the above-mentioned solution and incubated overnight at 37°C. Library plasmids isolated from His⁺ β-galactosidase⁺ colonies were amplified in *E. coli* after selection for ampicillin resistance. Library inserts were sequenced by using GAD-fw primer (Table 3). Positive interactions were confirmed by retransformation of yeast strain HF7c with the isolated library plasmid in combination with pGBDKc1.virD2.

Results

VirD2 protein localizes to the nucleus of S. cerevisiae.

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

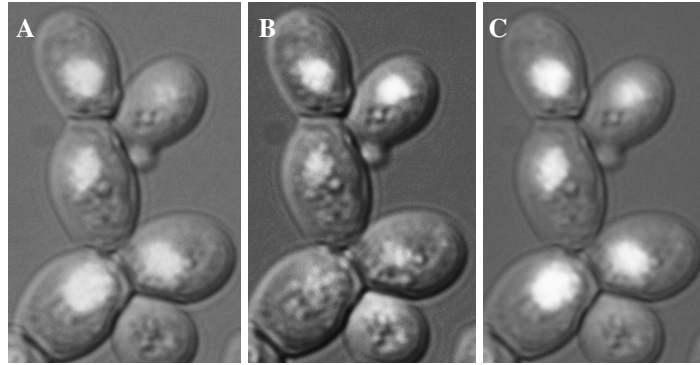


Figure 1. Subcellular localization of VirD2 fused at its N-terminus to C-terminus of GFP in *S. cerevisiae* strain CEN.pk113-3B. A, fluorescence microscopy of expressed GFP proteins. B, DAPI staining of the same cells. C, Superimposition of figures 1A and 1B. Cells are visualized by a Zeiss Axio-plan-2 imaging microscope.

VirD2 interacts with S. cerevisiae partners

To identify the *S. cerevisiae* proteins that interact with VirD2, we conducted a yeast two-hybrid screen using the bait plasmid pGBDKc1.*virD2* encoding VirD2 fused to Gal4 DNA-binding protein (Gal4 BD-VirD2). Gal4 BD-VirD2 alone could not activate the *HIS3* reporter gene of the HF7c strain. Yeast two hybrid screens were carried out by transformation of the HF7c strain, carrying pGBDKc1.*virD2*, with the *S. cerevisiae* genomic DNA libraries (James *et al.*, 1996.). From 5 transformations, 8.4×10^5 transformants were obtained, of which 670 were histidine positive. After re-streaking on selection media 19 histidine positive strains were left.

Sequence analyses of the yeast DNA inserts of the library plasmids isolated from these 19 strains revealed that only three contained an in-frame fusion with the *GAL4* activating domain. Transformation of the HF7c strain, carrying the empty bait vector pGBDKc1 (without *virD2*), with each of those 19 prey vectors showed that for transcription activation of the *HIS3* reporter, VirD2 was required as shown in figure 2 for two representative clones. In-frame fusions were found with *AGC1* (*YPR021C*, N-terminal 976 bp of the 2709 bp open reading frame), encoding a mitochondrial amino acid transporter; *CDC55* (*YGL190C*, C-terminal 846 bp of the 1581 bp open reading frame), encoding the non-essential regulatory subunit B of protein phosphatase 2A; and *YMR317W* (C-terminal 846 bp of the 3423 bp open reading frame), an ORF of unknown function. Surprisingly, among the 16 not-in-frame fusions DNA inserts containing parts of *HAP4*, parts of *MIG2* and parts of an rDNA region on the right

arm of chromosome XII (including *rDNA18*, *rDNA25*, *rDNA37*, *rDNA58*, the non-coding regions between them *ITS1*, *ITS2*, *NTS2* and *rDNA ARS1200-1*) were identified 2, 4 and 5 times, respectively. The list and function of the 19 identified DNA inserts are shown in Table 3. Figure 3 also shows the correspondence of the identified inserts to the yeast genes.

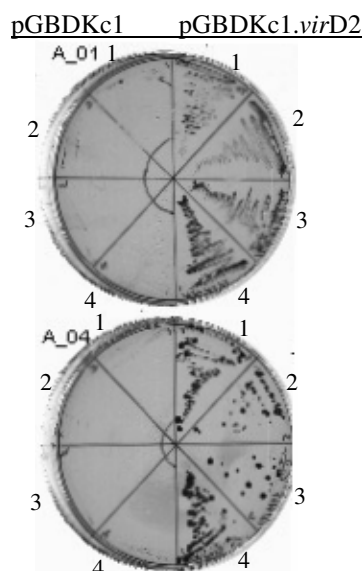
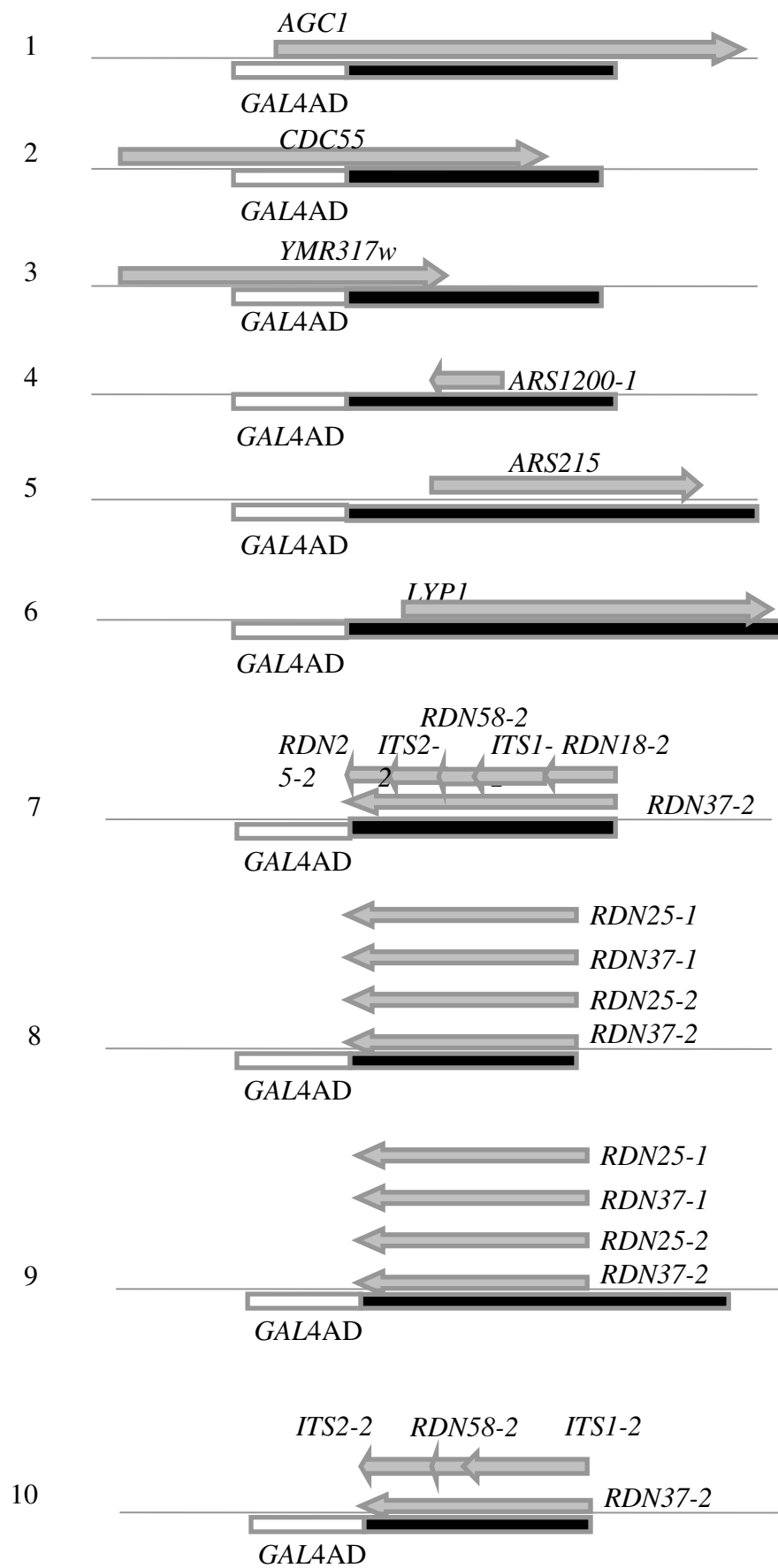


Figure 2. Dependency of *HIS3* expression in the yeast reporter strain HF7c on the presence of both *virD2* and identified clones. Two representative clones (A_01, *rDNA ARS1200-1*; A_04, *MIG2*) are shown. Four transformants (number 1-4) containing pGBDKc1-*virD2* and the isolated pGAD library plasmid are shown in the right hand part of the plate. Four transformants containing the empty pGBDKc1 and the isolated pGAD library plasmid are shown in the left hand part of the plate.

Table 3. Function of the 19 identified yeast DNA inserts that activate *HIS3* transcription in yeast two hybrid screens using *Agrobacterium* VirD2 as the bait.

Prey insert ^a	Times identified	Size of the insert(s) (bp)	In-frame fusion	Function ^a
<i>AGC1 (YPR021C)</i>	1	1100	Yes	Mitochondrial amino acid transporter
<i>ARS215</i>	1	3300	No	Autonomously Replicating Sequence
<i>CDC55 (YGL190C)</i>	1	1050 bp	Yes	Subunit B of protein phosphatase 2A
<i>HAP4 (YKL109W)</i>	2	1600, 850	No	Subunit of the HAP complex
<i>LYP1 (YNL268W)</i>	1	2050	No	A lysine permease
<i>MIG2(YGL209W)</i>	4	800, 1100, 1550, 2050	No	Multicopy Inhibitor of GAL gene expression
<i>MIG3 (YER028C)</i>	1	-	No	Probable transcriptional repressor
<i>rDNA ARS1200-1</i>	1	200	No	Autonomously Replicating Sequence
<i>RDN18,25,37,58, ITS1,2, and NTS2</i>	4	1050, 800, 1300, 800	No	rDNAs and the non-coding regions between them
<i>ROX1(YPR065W)</i>	1	200	No	Repressor of hypoxic genes
<i>TEA1 (YOR337W)</i>	1	1550	No	Ty1 enhancer activator
<i>YMR317W</i>	1	1037	Yes	Putative protein of unknown function

a) Gene systematic name and functions obtained from *Saccharomyces* Genome Database (www.yeastgenome.org)



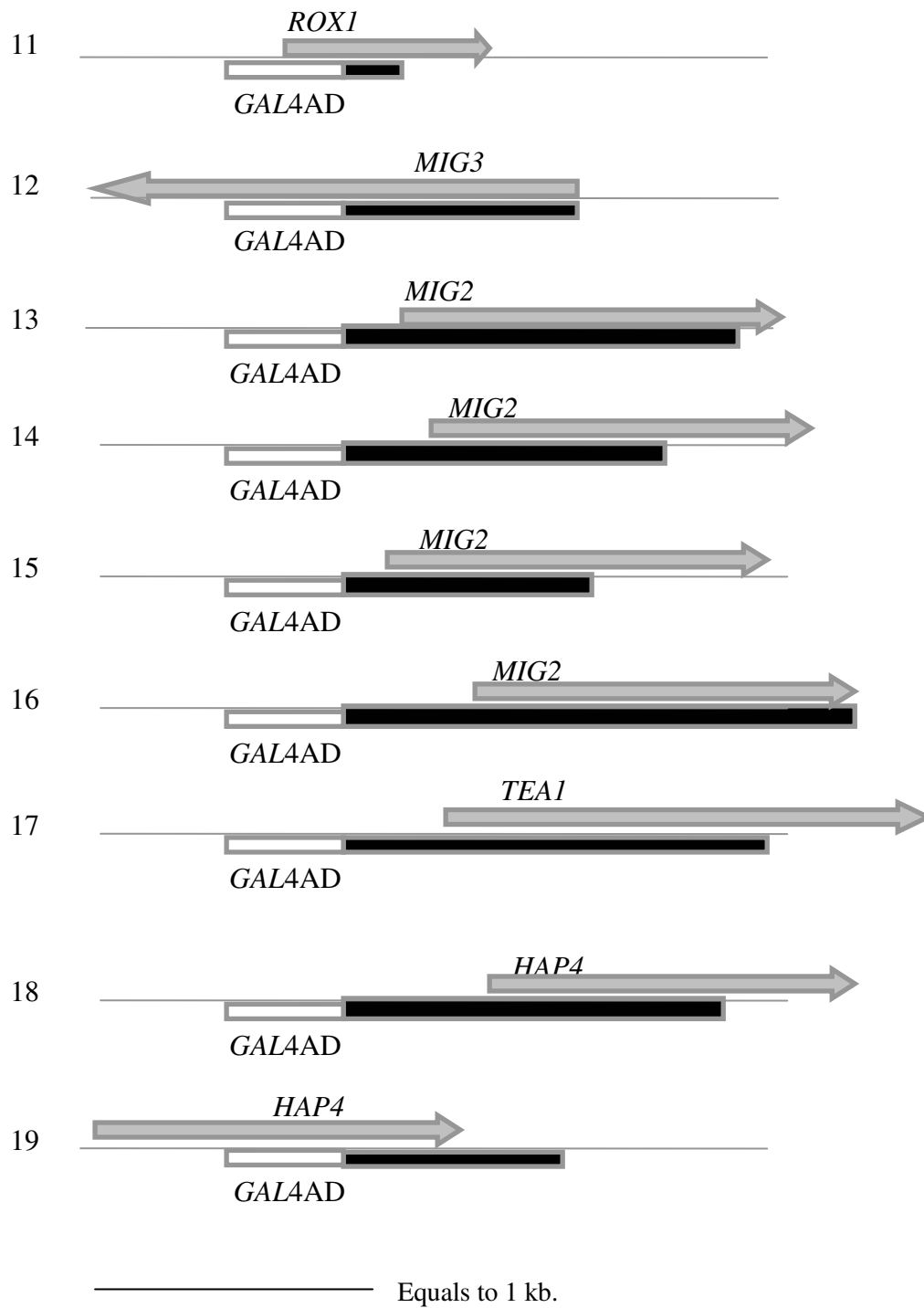


Figure 3. Presentation of the identified inserts that activate *HIS3* transcription in yeast two hybrid screens using *Agrobacterium* VirD2 as the bait.

Discussion

For a better understanding of the function of VirD2 in the yeast *S. cerevisiae* we analyzed the subcellular localization of VirD2 expressed in yeast. 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) 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 *S. cerevisiae*. C-terminal and N-terminal fusions of the *virD2* to GFP were expressed in yeast cells to visualize the subcellular localizations of them. Indeed, VirD2 fused at its N-terminus to GFP localized to the nuclei of *S. cerevisiae* (Fig. 1). Nuclear localization of VirD2 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 only background fluorescence was seen in our experiments. 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 yeast and 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 may indicate 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.

In a yeast two hybrid screen to identify the *S. cerevisiae* proteins that bind to the VirD2 protein, three proteins encoded by *AGC1*, *CDC55* and *YMR317W* were found. Moreover, inserts of *HAP4*, *MIG2*, and parts of an rDNA region on the right arm of chromosome XII (containing rDNA18, rDNA25, rDNA37, rDNA58 and the non-coding regions between them, *ITS1*, *ITS2*, *NTS2* and rDNA *ARS1200-1*) were identified 2, 4 and 5 times, respectively, although without in-frame fusions to *GAL4* AD. It has to be realized that the results of this two hybrid screen are preliminary. Further research is needed to show whether the identified interactions are relevant. These interactions still have to be confirmed by independent techniques like co-immunoprecipitation and *in vitro* protein interaction studies.

Agc1 protein is a mitochondrial carrier protein which has three tandem repeats of Solcar (Solute carrier repeat) domain in its C-terminal part. The functional significance of this putative binding is unclear.

Cdc55 is a subunit B of protein phosphatase 2A which has two protein phosphatase 2A regulatory subunit (PR55) signature sequences and a Trp-Asp (WD) repeat signature (<http://www.expasy.org>). It has already been shown *in planta* that *Agrobacterium* uses the host regulatory phosphorylation-dephosphorylation system to deliver VirD2 to the plant nucleus. While phosphorylation of VirD2 by nuclear cyclin-dependent kinase-activating kinases (Cak2M) from alfalfa and *Arabidopsis* enhances its nuclear import (Bako *et al.*, 2003), dephosphorylation of it by the type 2C serine/threonine phosphatase Dig3 partially inhibits nuclear import of VirD2 in tobacco BY2 protoplasts (Tao *et al.*, 2004). Hence, the interaction of VirD2 with yeast *Cdc55* phosphatase may represent a conserved regulatory mechanism of its nuclear import in eukaryotic host cells. Alternatively, since *Cdc55* is a nuclear protein and has multiple functions in mitosis it may facilitate the integration of T-DNA through interaction with VirD2.

YMR317W encodes a protein of unknown function. It has been shown that the protein interacts with Ino4 in a yeast two hybrid screen (Uetz, P., *et al.*, 2000). Ino4 is a

transcription factor required for derepression of inositol-choline-regulated genes involved in phospholipid synthesis (Santiago and Mamoun, 2003) and is also linked to hypoacetylation in *S.cerevisiae* (Pham *et al.*, 2007). The possible link of Ymr317w to hypoacetylation in yeast through its interaction with Ino4 may further emphasize the importance of histone acetylation/deacetylation of host cell on AMT (Chapter 4).

Although not in-frame fused with the *GAL4* AD, the DNA inserts of *MIG2* and *MIG3* were identified four and one times in our screens. Their effect on the expression of the *HIS3* reporter gene is dependent on the presence of VirD2. Therefore, we suggest that VirD2 is in some way influencing or interacting with the *MIG2* and *MIG3* genes. As *MIG* genes have been isolated as a multicopy inhibitor of galactose (*GAL*) gene expression, disruption of their function may cause increased expression of *GAL* genes including *GAL*-promoter driven reporter genes. All four inserts of *MIG2* found contain parts of the promoter and N-terminal part of *MIG2* and *MIG3* (Fig 2). However, the mechanism of how this part of *MIG2* together with VirD2 influences transcription is unclear. We also identified *HAP4*, not in frame fused to the *GAL4* AD. Hap4 is a transcriptional activator and global regulator of respiratory gene expression. Deletion of the *HAP4* gene results in a decreased AMT (Chapter 3).

Surprisingly, DNA inserts of an rDNA region on the right arm of chromosome XII (containing rDNA18, rDNA25, rDNA37, rDNA58 and the non-coding regions between them, *ITS1*, *ITS2*, *NTS2* and rDNA *ARS1200-1*) were identified in our screen five times. Moreover, an autonomously replicating sequence, *ARS215*, *LYP1* (*YNL268W*), encoding a lysine permease, *ROX1* (*YPR065W*) encoding a heme-dependent repressor of hypoxic genes, and *TEA1* (*YOR337W*) encoding a Ty1 enhancer activator are also identified in our screen without in-frame fusions to *GAL4* AD. The relation between these inserts and VirD2 remain unclear.

Using two hybrid screens for plant proteins interacting with VirD2, importin α (Ballas *et al.*, 1997) and cyclophilin-related proteins (Deng *et al.*, 1998; Bako *et al.*, 2003; A. Briancon-Marjollet and H. van Attikum (unpublished)) have been identified. Although *AGC1*, *CDC55* and *YMR317W* have homologous genes in *Arabidopsis* (with 53-61% homology) none of them is reported to interact with VirD2. We screened approximately 8.4×10^5 clones of yeast DNA library and didn't find any yeast homologs of the plant proteins interacting with VirD2. As the library consists of

7.8×10^5 independent clones, additional screening may be required to identify these VirD2 interaction partners. Deletion of *AGC1* and *YMR317W* resulted in a similar AMT as the wild type strain in our screen (Chapter 3, data not shown) indicating that these genes are not essential for AMT.

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