VirD2 of Agrobacterium tumefaciens : functional domains and biotechnological applications

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Chapter 3

The DUF domain of VirD2 determines recruitment of VirD2 to the Type 4 Secretion System within *Agrobacterium tumefaciens*

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Abstract

VirD2 is the *Agrobacterium tumefaciens* protein responsible for T-strand processing. Besides its relaxase domain, it contains a large domain of unknown function. In a previous study we have shown that this DUF domain can be replaced by the very short C-terminal Type 4 Secretion System translocation sequence of the unrelated protein VirF. We have confirmed this result here in *Nicotiana glauca* and *Kalanchoë daigramontiana*. Also, we have studied the subcellular localization of GFP-tagged VirD2 constructs. In the absence of DUF, the VirD2 protein is randomly distributed in the bacterium, but that full length VirD2 shows a localization at the cell poles in more than 60% of the cells. Polar localization is determined for the most part by the N-terminal 60 amino acids of DUF. Surprisingly, the short C-terminus of VirF is able to substitute for DUF in targeting truncations of VirD2 to the cell poles. Polar localization of VirD2 truncations correlates with the virulence of the corresponding *Agrobacterium* strain.

Introduction

Agrobacterium tumefaciens causes crown gall disease in dicotyledonous plants by transferring some of its own DNA (the transferred or T-DNA) into these plants, where it integrates into the plant's genome. The T-DNA, situated on the large tumor-inducing plasmid (pTi) contains genes causing the plant cells to divide rapidly, forming a tumorous overgrowth, and to produce opines, which *Agrobacterium* can use as carbon source (recently reviewed in Pitzschke and Hirt, 2010). In the laboratory, it was shown that *Agrobacterium* can mediat DNA transfer also to monocotyledonous plants (Hooykaas – van Slogteren *et al.*, 1991), yeast and other fungi (Bundock *et al.*, 1995, de Groot *et al.*, 1998).

The T-DNA is delimited by a 24 basepair direct repeat: the left and right border (LB and RB) sequences (Yadav *et al.*, 1982). After it was discovered that any DNA sequence present between the LB and RB would be transferred to the plant recipient, the binary vector system was developed, in which the LB and RB are present on an extra plasmid, instead of on the large (~200kb) pTi, which is difficult to manipulate (Hoekema *et al.*, 1983). Since then, *Agrobacterium*-mediated transformation (AMT) has become one of the

preferred methods of plant and fungal transformation.

AMT starts upon the perception of phenolic compounds, released by wounded plant cells, by *Agrobacterium*. This triggers a signaling cascade which results in expression of bacterial virulence (*vir*) genes (reviewed in Zhu *et al.*, 2000, Pitzschke and Hirt, 2010). For T-DNA processing, the VirD1 and VirD2 proteins are essential. VirD2 is a relaxase, able to nick the border sequences to release a single-stranded DNA molecule called the T-strand. The VirD1 protein is supposed to aid by local unwinding of the DNA (Jayaswal *et al.*, 1987, Lessl and Lanka, 1994, Scheiffele *et al.*, 1995). After processing, VirD2 remains covalently attached to the 5' end of the T-strand to form the T-complex (Dürrenberger *et al.*, 1989, Scheiffele *et al.*, 1995, Vogel and Das, 1992, reviewed in Lanka and Wilkins, 1995, and Gelvin, 2000). VirD2 is, as such, responsible for the translocation of the T-complex to the recipient cell (Van Kregten *et al.*, 2009, Llosa *et al.*, 2002). Translocation occurs via the Type IV Secretion System (T4SS) formed by the VirB1-11 proteins and the coupling factor VirD4 (reviewed in e.g. Christie, 2004, Llosa *et al.*, 2002).

Apart from the T-complex, other Vir proteins like VirE3 (Schrammeijer *et al.*, 2003), VirE2 and VirF (Vergunst *et al.*, 2000), and VirD5 (Vergunst *et al.*, 2005) are translocated to the recipient species during AMT. All these translocated proteins probably interact directly or indirectly with the VirD4 protein, which is the first to act in the process of translocation (Cascales and Christie, 2004). Since the VirD4 protein (Kumar and Das, 2002) and the VirB proteins forming the T4SS proper (Judd *et al.*, 2005) are found at one or both poles of the rod-shaped *Agrobacterium* cells, polar localization of transport substrates might be expected as well. Recently, using deconvolution microscopy, components and substrates of the T4SS were found to be located around the perimeter of the cell in a helical fashion, rather than at the poles (Aguilar *et al.*, 2010). For VirE2, which was shown to interact with VirD4 in an immunoprecipitation assay, polar localization has indeed been demonstrated, but to our knowledge this has not been demonstrated for other translocated Vir proteins. Although attempts have been made, an interaction between VirD2 and VirD4 has not yet been found, suggesting that their interaction is weak or that another binding partner is involved (Atmakuri *et al.*, 2003).

In a previous study (Van Kregten et al., 2009), we have focused on the functional properties of the large domain of unknown function (DUF) which, except for some short terminal sequences, forms the larger part of C-terminal half of VirD2 (Fig. 1). The DUF is relatively unstructured and is poorly conserved between VirD2 proteins from related *Agrobacterium* species (Howard *et al.*, 1992). As we demonstrated, the N-terminal half of

VirD2, consisting of the relaxase domain, can successfully be used for AMT of *Arabidopsis thaliana* root explants when supplemented with the C-terminal T4SS translocation signal of VirF (VirD2-204-F). This means that the DUF domain and the C-terminal sequences, in total about half of the protein, can be replaced by a very short (37 amino acids) sequence from a protein that is not involved in T-DNA processing. However, when only short C-terminal sequences of VirD2 were fused to the relaxase domain VirD2-204, such as combinations of the nuclear localization signal (NLS) (Rossi *et al.*, 1993), the arginine-rich putative T4SS translocation signal (Vergunst *et al.*, 2005), and the omega (ω) sequence (Bravo-Angel *et al.*, 1998, Mysore *et al.*, 1998, Shurvinton *et al.*, 1992), AMT remained severely hampered. Remarkably, AMT was restored when a stretch of amino acids corresponding to residues 205 to 264 of VirD2 was fused in between the relaxase domain and the short C-terminal VirD2 sequence, providing compelling evidence that these 60 amino acids play an important role in AMT within the native VirD2 protein (Van Kregten et al., 2009).

Figure 1: Schematic overview of the domains of VirD2. The N-terminal half of VirD2 consists of the relaxase domain (Scheiffele *et al.*, 1996, Steck *et al.*, 1990). The C-terminal half encompasses the domain of unknown function (DUF), of which DUF-60 is functionally important (Van Kregten *et al.* 2009). The C-terminal domain consists of a bipartite NLS, the arginine-rich sequence important in translocation, and the ω sequence (Howard *et al.*, 1992, Vergunst *et al.*, 2005, Shunvinton *et al.*, 1992).

In the present study, we wanted to investigate whether the importance of the 205-264 region of the DUF domain (DUF-60) for AMT was also evident when using other plant species than *Arabidopsis thaliana*. In addition to that, we wanted to gain more insight in the precise function of the DUF-60 domain. The VirD2 region downstream of amino acid residue 204 is not required for the relaxase reaction (Scheiffele *et al.*, 1995, Steck *et al.*, 1990, Van Kregten et al., 2009), but it may be involved in contacting elements of the T4SS

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or in recruitment to this system. However, no interaction between VirD2 and VirD4, the coupling protein of the T4SS, has been found (Atmakuri *et al.*, 2007), but through its DUF-60 domain may still interact with another component of the T4SS and thus be involved in polar localization of the T-complex.

Our current results provide evidence that the DUF-60 function is not restricted to AMT of *Arabidopsis*, but can also be observed in AMT of *Nicotiana glauca* and *Kalanchoë daigramontiana*. By using GFP-tagged VirD2 constructs, we found that DUF-60 is crucial for polar localization of GFP-tagged VirD2. The short T4SS translocation signal of VirF can substitute for DUF-60 in inducing polar localization of VirD2. The relevance of these findings is discussed.

Results

DUF-60 can be replaced by the C-terminal end of VirF in plant transformation

The DUF domain of VirD2 is dispensable for transformation of *Arabidopsis* when the T4SS translocation signal of VirF is fused to C-terminally truncated versions of VirD2, such as VirD2-204 (Van Kregten *et al.*, 2009). Fusion of the native C-terminal end of VirD2 to VirD2-204 resulted in only a very low transformation efficiency, but addition of DUF-60 (60 amino acids located adjacent to amino acids 204) in between VirD2-204 and the VirD2-derived C-terminal peptide restored transformation efficiency to normal levels (Van Kregten *et al.*, 2009). Apparently, the C-terminal translocation signal of VirD2 needs assistance from DUF-60 to fulfill its function.

To investigate if the effect of DUF-60 in other plant species is similar to the effect observed in *Arabidopsis*, several VirD2 truncations were made, all N-terminally tagged with the FLAG tag and the SV40 NLS (omitted in names of constructs for brevity). The FLAG tag was used for easy detection of protein expression and the SV40 NLS to ensure nuclear localization. A schematic overview of the constructs used in this study is shown in Fig. 2. We created VirD2-204, in which the C-terminal half is completely deleted, and VirD2-264, in which DUF-60 is included, but the rest of the C-terminus is deleted. These constructs were also supplemented with elements of the C-terminal end of VirD2, to create VirD2-CtermΔω and VirD2-264-CtermΔω. This C-terminal end lacks the 5 C-terminal amino acids, but is sufficient to enable translocation (Van Kregten *et al.*, 2009). The constructs
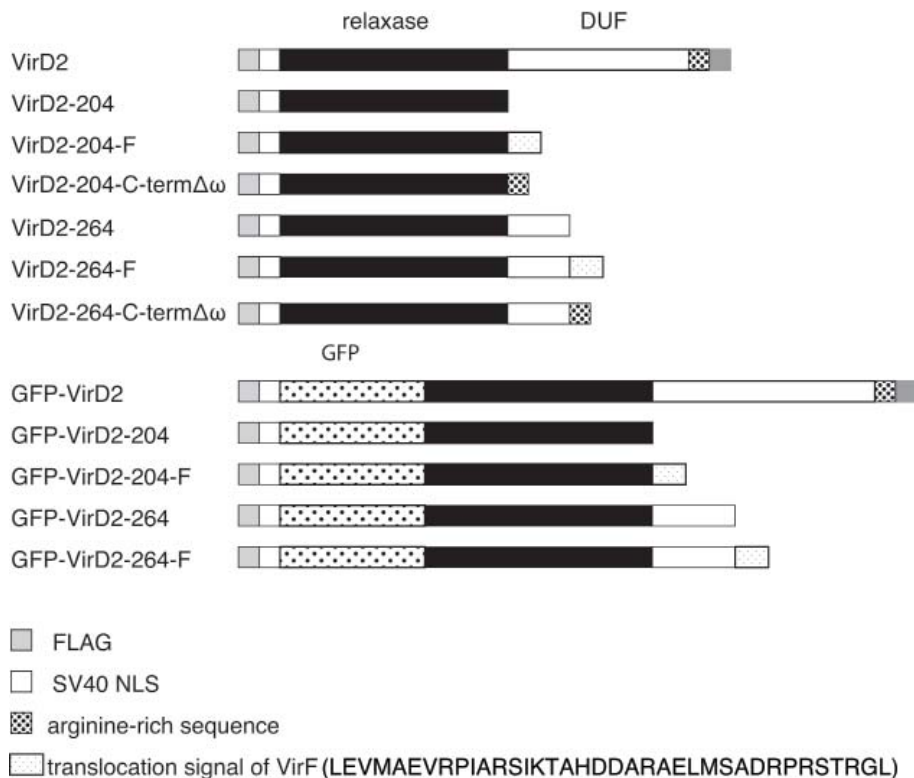
Figure 2: A schematic overview of the proteins used in this study.

were also supplemented with the T4SS translocation signal of VirF, to create VirD2-204-F and VirD2-264-F.

We inoculated *Nicotiana glauca, Kalanchoë daigramontiana, Solanum lycopersicum* and *Helianthus annuus* with *Agrobacterium* strain LBA2569 (Δ*virD2*, ΔT-DNA, Vergunst, Den Dulk-Ras and Hooykaas, unpublished) expressing the relevant VirD2 truncations or LBA1010 as positive control (wild type strain, Koekman *et al.*, 1982). Tumor formation was photographed after three (*N. glauca*) or six (*K. daigramontiana*) weeks. *H. annuus* and *S. lycopersum* only formed tumors when inoculated with the wild type control *Agrobacterium* strain (data not shown).

Agrobacterium strains that expressed the VirD2 truncations VirD2-204 or VirD2-264 induced no tumor formation in *N. glauca* or *K. daigramontiana*. Tumors were induced by *Agrobacterium* strains expressing VirD2-204-F and VirD2-264-F, the truncations that were complemented with the C-terminal 37 amino acids of VirF. An *Agrobacterium* strain expressing VirD2-264-CtermΔω caused smaller tumors than strains expressing VirD2-

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204-F and VirD2-264-F. An *Agrobacterium* strain expressing VirD2-204-CtermΔω caused smaller tumors than an *Agrobacterium* strain expressing VirD2-264-CtermΔω (Fig. 3, Table 1). Apparently, as previously found for *Arabidopsis* root transformations (Van Kregten *et al.*, 2009), the C-terminal sequence of VirF can also replace the C-terminal half of VirD2 in tumor formation on other plant species. Hence, just as for *Arabidopsis*, the DUF domain of VirD2 is not required for transformation of *N. glauca* or *K. daigramontiana*. By combining our previous results with the newly gathered data, it seems that DUF does not play a role as host range factor in the tested plant species.
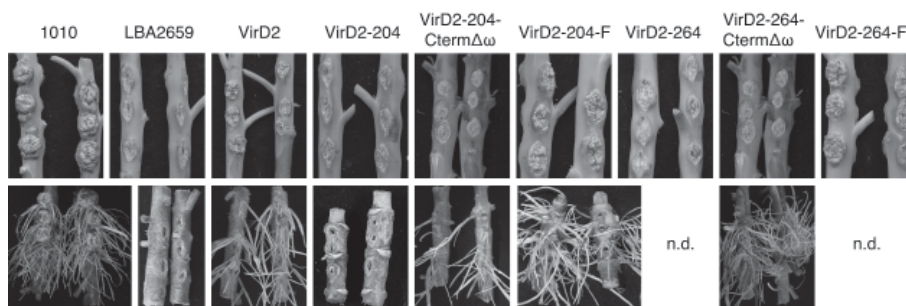
Figure 3: Tumor assay in *Nicotiana glauca* (top) and *Kalachoë tubiflora* (bottom). LBA1010 is a wild type *Agrobacterium* strain, LBA2569 is LBA1010Δ*virD2*, and is used for the expression of VirD2 mutants. All VirD2 constructs are N-terminally tagged with FLAG and NLS. Left to right: LBA1010, LBA2569, LBA2569 expressing VirD2, LBA2569 expressing VirD2-204, LBA2569 expressing VirD2-204-CtermΔω, LBA2569 expressing VirD2-204-F, LBA2569 expressing VirD2-264, LBA2569 expressing VirD2-264-CtermΔω, LBA2569 expressing VirD2-264-F Tumors were photographed after 4 weeks (*N. glauca*) and 6 weeks (*K. daigramontiana*). n.d.: not determined.

DUF-60 and the C-terminus of VirF direct GFP-VirD2 to the cell poles

Since our results pointed towards a function of DUF-60 as part of the T4SS translocation signal within *Agrobacterium* cells, we went on to investigate if DUF-60 may function as a factor influencing recruitment of VirD2 to the T4SS. We thus fused GFP to the VirD2 truncations in order to visualize the subcellular localization of these proteins in *virD2* deleted *Agrobacterium* strain LBA2569. GFP-VirD2, GFP-VirD2-204, and GFP-VirD2-264 were expressed under control of the *virF* promoter. As control, free GFP was expressed in *Agrobacterium* strain LBA1100 (ΔT-DNA, Beijersbergen *et al.*, 1992), from plasmid pSDM1761 (Bloemberg *et al.*, 2000). After induction of the *Agrobacterium* cells

Table 1

construct[a]	plant species	
	N. glauca	*K. daigramontiana*
LBA1010	+++	+++
LBA2569	-	-
VirD2	+	+
VirD2-204	-	-
VirD2-204-CtermΔω	+/-	+/-
VirD2-204-F	++	++
VirD2-264	-	n.d.[b]
VirD2-264- CtermΔω	+	+
VirD2-264-F	++	n.d.

[a]LBA1010 is a wild type *Agrobacterium* strain, LBA2569 is LBA1010Δ*virD2*. All VirD2 constructs are N-terminally tagged with FLAG and NLS. Tumor formation was scored after 3 (*N. glauca*) or 6 (*K. daigramontiana*) weeks.
[b]n.d.: not determined

with acetosyringone, localization of the fluorescent signal was determined by confocal microscopy.

As shown by the results in Table 2 and Figure 4, a control strain expressing free GFP showed fluorescent signal distributed throughout the entire cell. Cells expressing GFP-VirD2 showed polar localization in 63% of cells. Cells expressing GFP-VirD2-204, from which DUF is deleted, showed a dramatic drop in polar localization, to only 8%. In cells expressing GFP-VirD2-264, polar localization is restored to 50% of cells. Apparently, DUF-60 is very important for polar localization of VirD2.

The addition of the C-terminus of VirF restores to a large extent the ability of VirD2 truncations to mediate transformation of *Arabidopsis* root cells (Van Kregten *et al.*, 2009). To investigate if the C-terminal T4SS translocation signal of VirF, consisting of only 37 amino acid residues, is also sufficient to restore the normal pattern of polar localization of VirD2 truncations in induced *Agrobacterium* cells, we expressed GFP-VirD2-204-F in *Agrobacterium* strain LBA2569 and determined its subcellular localization. As shown in Table 2 and illustrated in Fig. 4, the addition of the C-terminal 37 amino acids of VirF to GFP-VirD2-204 restored the amount of cell showing polar localization to 41%. When both DUF-60 and the C-terminus of VirF are present, the percentage of polar localization of GFP-VirD2-264-F is restored to 62%, the level also found for GFP-VirD2. However, cells expressing GFP-VirD2-264-F do not show neat localization to the cell poles, but had

a somewhat spotted phenotype. Apart from fluorescent signal at the cell poles, they had additional spots in other places in the cell (e.g. the middle cell in the picture of GFP-VirD2-264-F in Fig. 4).
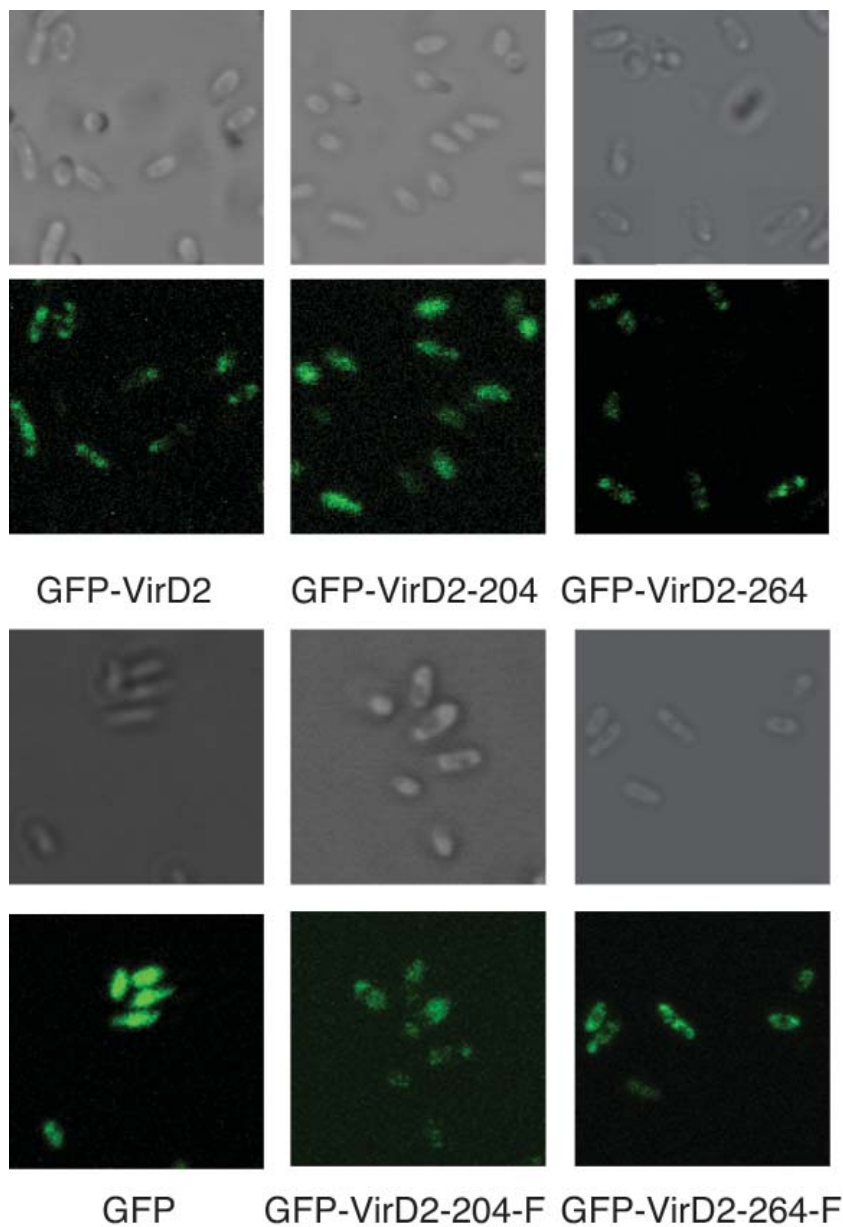

Figure 4: Localization of GFP-tagged VirD2 truncations in *Agrobacterium*. Upper pictures: visual field, lower pictures: fluorescence. The proteins produced in *Agrobacterium* strain LBA2569 (Δ*virD2*) are indicated below the images.

In summary, the data regarding the localization of VirD2 established that DUF-60 is crucial for determining polar localization of VirD2 within *Agrobacterium* cells. When DUF-60 is lacking, almost all polar localization disappears. The short C-terminal sequence of VirF can compensate for the lack of DUF-60, restoring the polar localization of VirD2. These results are discussed below.

Table 2: subcellular localization of VirD2 truncations in *Agrobacterium*

Agrobacterium strain expressing	percentage of cells showing:	
	polar localization*	signal in entire cell
GFP	0	100
GFP-VirD2-204	8	92
GFP-VirD2-264	50	50
GFP-VirD2	62	38
GFP-VirD2-204-F	41	59
GFP-VirD2-264-F	62**	38

* For every strain, at least 100 cells were scored.
** mostly with "spotted" phenotype

Discussion

Agrobacterium's VirD2 protein is remarkably multifunctional. It is responsible for the processing of the T-DNA (Jayaswal *et al.*, 1987, Lessl and Lanka, 1994, Scheiffele *et al.*, 1995). It is also responsible for the translocation of itself and the T-strand, to which it is covalently bound, through the T4SS of *Agrobacterium* into the cytosol of the recipient cell (Van Kregten *et al.*, 2009, Llosa *et al.*, 2002). Furthermore, it plays a role in nuclear import of the complex (reviewed in Gelvin, 2010).

In this study, we have further investigated the function of the large DUF domain in VirD2, making up almost half of its size (Fig. 1) and thus far a part of VirD2 to which a clear function has yet to be assigned. We focused on DUF-60, the N-terminal 60 amino acids of DUF, since a possible function of DUF seems to be contained within this region (Van Kregten *et al.*, 2009).

Our results concerning tumor formation in *N. glauca* and *K. daigramontiana* (Fig. 3 and Table 1) correlate with previous results obtained in *Arabidopsis* (Van Kregten *et al*, 2009). In all plant species tested so far, the requirements for successful transformation

are either DUF-60 and the C-terminal end of VirD2, or the C-terminus of VirF that are attached to the relaxase domain. The fact that the C-terminus of VirF can substitute for DUF-60 and the C-terminal end of VirD2 in very different plant species argues against a subtle function of DUF-60 in recipient plant cells. In case such a subtle function would exist, for instance via interaction with a particular host protein, different plant species would be expected to react differently upon the presence of DUF-60. For the VirF protein, such a host dependent attribution to virulence has been described (Hooykaas *et al.*, 1984, Tzfira *et al.*, 2004). The data presented here and previously strongly suggest that this is not the case for DUF-60. Obviously, just testing three different plant species would not suffice for drawing a definitive conclusion that DUF-60 does not exert any function in some plant species, had we not discovered that the function of DUF-60 lies within *Agrobacterium* cells.

If DUF-60 is important for the transformation process, but not required for a function in the host plant species, it is logical to assume it fulfills a function within *Agrobacterium* cells. Since the relaxase reaction does not require DUF, we focused our efforts on the subcellular localization of VirD2. A likely function of DUF-60 is recruitment of the T-complex to the T4SS. Since the T4SS is located at the poles of *Agrobacterium* cells, DUF-60-dependent polar localization would provide evidence for this hypothesis. Indeed, we found that deletion of DUF resulted in an almost complete loss of polar localization: in cells expressing GFP-VirD2-204, polar localization dropped to 8% of the cells, wherein GFP-VirD2 localized to the cell poles in 62% of cells (Table 2, Fig. 4). Our results on GFP-VirD2 are comparable to results found by other groups who found polar localization in 55% (Guo *et al.*, 2007b), and between 50 to 70% polar localization (Atmakuri *et al.*, 2007) of *Agrobacterium* cells expressing GFP-VirD2. In a recently published article, T4SS components and substrates were shown to be positioned in a helical array around the circumference of the cell, rather than at the cell poles (Aguikar *et al.*, 2010). It would be interesting to apply deconvolution microscopy, the technique used by Aguilar and co-workers, to the strains used in this study, especially for further investigation of GFP-VirD2-264, since the 'spotted' phenotype observed in strains expressing this construct is reminiscent of such helical arrays.

Polar localization was predominantly determined by DUF-60, as shown by construct GFP-VirD2-264, which displayed polar localization in 50 % of cells. We cannot exclude that a larger domain than DUF-60 mediates polar localization; however, a construct expressing GFP-DUF-60, which could theoretically demonstrate a modular DUF-60 potential for polar localization, proved to be toxic for *E. coli* cells, and could therefore not be prepared.

For all VirD2 truncations tested, the extent of polar localization of VirD2 correlated

with virulence of the host bacterium: constructs displaying polar localization and containing a T4SS translocation signal can be translocated and can cause transformation, whereas those not locating to the poles do not mediate transformation (Tables 1 and 2, Fig. 3 and 4, and Van Kregten *et al.*, 2009). This strongly indicates that polar localization is required for translocation via the T4SS of *Agrobacterium*.

Apparently, the translocation of VirD2 is a two-step process: recruitment of VirD2 to the cell poles and subsequent interactions with the T4SS resulting in translocation. For polar recruitment, DUF-60 is required, while for translocation to a recipient cell, sequences of the C-terminal end of VirD2 are required in addition. As can be inferred from our data, the short and continuous stretch of 37 C-terminal amino acids of VirF suffices for both polar localization and translocation. To our knowledge, possible interaction partners of VirF within *Agrobacterium* cells have never been described, so the mechanism of polar localization of VirF is currently unknown. In addition to that, not much is known about the recruitment to the T4SS of other translocated Vir proteins either. Only for VirE2 it was found that this protein interacts directly with the coupling protein VirD4 at the cell poles. If VirD4 is deleted, polar localization of VirE2 is lost as well (Atmakuri *et al.*, 2003). No data is available on domain requirements of VirE2 for interaction with VirD4. In a related system, the requirements for translocation of the Bep proteins of *Bartonella henselae* seem comparable to those of VirD2. An T4SS system largely similar to that of *Agrobacterium* exists in *Bartonella henselae*: the requirements for translocation of the effector proteins BepA-G are the presence of the Bep intracellular delivery (BID) domain and a C-terminal sequence rich in charged amino acids, which serves as T4SS translocation signal. Together, these two domain serve probably as a bipartite translocation signal (Schulein *et al.*, 2005).

To explain differences in requirements for translocation between VirD2 and VirF, we hypothesize that the presence of an extra domain required for translocation of VirD2 must serve an extra purpose as well. We propose that DUF-60 serves as an interaction domain. Several interactions between VirD2 and *Agrobacterium* proteins have been described. VirD2 interacts with VirC1 and the <u>V</u>irD2-<u>B</u>inding <u>P</u>roteins 1-3 (VBP1-3) (Atmakuri *et al.*, 2007, Guo *et al.*, 2007a). For all these factors, loss of interaction with VirD2 has been reported to result in loss of polar localization of VirD2 (Atmakuri *et al.*, 2007, Guo *et al.*, 2007a, Guo *et al.*, 2007b). Both VirC1 and VBP interactions seem equally required for polar localization. We hypothesize that DUF-60 represents a binding site for one or more of these interactants. The site of interaction between VirD2 and VirC1 has not been mapped yet, but VBP1 was shown to interact with the N-terminal half of VirD2, upstream of a *Bam*HI restriction site

within the VirD2 encoding region of the octopine-type VirD2 (Guo *et al.*, 2007a). This includes DUF-60. Of VirC1, it was shown that it not only interacts with VirD2, but also binds to the overdrive sequence on the Ti plasmid (Toro *et al.*, 1989). By these interactions, VirC1 could coordinate the T-strand processing reaction, ensuring that VirD2 can only be translocated after having processed a T-DNA by linking the T-strand processing reaction to polar localization.

We propose that, possibly with some extension into the relaxase domain, DUF-60 is the T4SS recruitment domain of VirD2. In that manner, DUF-60 mediates the polar localization of VirD2 due to its interaction with VirC1 and/or the VBP proteins. We propose that VirD2 has two domains for translocation; DUF-60, controlling recruitment of the protein to the T4SS, and a translocation signal, controlling the actual translocation process.

Acknowledgements

Materials & methods

Bacterial strains and plasmids

Cloning steps were perfomed in *E. coli* strain DH10B. *Agrobacterium tumefaciens* wild type strain LBA1010 (Koekman *et al.*, 1982) and Δ*virD2* strain LBA2569 (C58, pTiB6, Δ*virD2*; Vergunst, A., Den Dulk-Ras, A. and Hooykaas, P.J.J., unpublished) were used for tumor formation assays. LBA2569 and LBA1100 (ΔT-DNA, Beijersbergen *et al.*, 1992) were used for microscopy studies.

Constructs used for tumor assays were described previously (Van Kregten *et al.*, 2009). For microscopy analysis, GFP was amplified from pEGFP (a gift from dr. De Boer, Add2X Biosciences) using primers GFP fw (GAATGCTCGAGGTGAGCAAGGGCGAGGAGCTG) and GFP rv (CTTACCTCGAGCTTGTACAGCTCGTCCATGCC). The product was digested with *Xho*I and ligated to similarly digested pSKN VirD2C (Van der Zaal, unpublished), to yield pSKN-GFP-VirD2. pSKN-GFP-VirD2 was digested with *Xmn*I and *Nde*I and the fragment of interest was ligated to similarly digested pSKN-VirD2-204 and pSKN-

VirD2-264 (described in Van Kregten *et al.*, 2009) to yield pSKN-GFP-VirD2-204 and pSKN-GFP-VirD2-264.

All pSKN plasmids were digested with *Not*I, and fragments containing the genes-of-interest of the plasmids were ligated to similarly digested pBFF or pBFFstop (an *Agrobacterium* expression vector, allowing expression of the gene of interest under control of the acetosyringone-inducible *virF* promoter; described in Van Kregten *et al.*, 2009), to yield pBFF-GFP-VirD2, pBFF-GFP-VirD2-204, pBFF-GFP-VirD2-264, and pBFFstop-GFP-VirD2, pBFFstop-GFP-VirD2-264, pBFFstop-GFP-VirD2-264. A schematic drawing of the proteins can be found in Fig. 2.

Microscopy

All constructs were electroporated into *Agrobacterium* strain LBA2569. Fluorescence was examined in cells from fresh overnight cultures induced with 200 µM acetosyringone. Aliquots of overnight cultures were resuspended in 0.9% NaCl solution, and visualized using a Zeiss Imager confocal microscope.

Tumor assay

Three- to four-week-old seedling of *Nicotiana glauca, Kalanchoë daigramontiana*, *Solanum lycopersum* and *Helianthus annus* were inoculated with 20 µL of an *Agrobacterium tumefaciens* suspension in physiological salt solution, at an OD of 0.8, from a fresh overnight culture. Tumors were photographed after four weeks (*N. glauca*), or six weeks (other species).

References

Aguilar, J., Zupan, J., Cameron, T.A. and Zambryski, P.C. 2010. *Agrobacterium* Type IV Secretion System and its substrates form helical arrays around the circumference of virulence-induced cells. *Proc. Natl. Acad. Sci. USA.* 107(8):3758-3763.

Atmakuri, K., Cascales, E., Burton, O.T., Banta, L.M., and Christie, P.J. 2007. *Agrobacterium* ParA/ MinD-linked VirC1 spatially coordinates early conjugative DNA transfer reactions. EMBO (Eur. Mol. Biol. Organ.) J. 26(10):2540-2551.

Atmakuri, K., Ding, Z., and Christie, P.J. 2003. VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. Mol. Microbiol. 49(6):1699-1713.

Beijersbergen, A., den Dulk-Ras, A., Schilperoort, R.A., and Hooykaas, P.J.J. 1992. Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science. 256(5061):1324-1327.

Bloemberg, G.V., Wijfjes, A.H., Lamers, G.E., Stuurman, N., and Lugtenberg, B.J. 2000. Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Mol Plant-Microbe Interact. 13(11):1170-1176.

Bravo-Angel, A.M., Hohn, B., and Tinland, B. 1998. The omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*. Mol. Plant-Microb. Interact. 11(1):57-63.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A., and Hooykaas, P. J.J. 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO (Eur. Mol. Biol. Organ.) J. 14(13): 3206-3214.

Cascales, E., and Christie, P.J. 2004. Definition of a bacterial type IV secretion pathway for a DNA substrate. Science. 304(5674):1170-1173.

Christie, P.J. 2004. Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. Biochem. Biophys. Acta. 1693(1-3):219-234.

De Groot, M. J., Bundock, P., Hooykaas, P. J.J., and Beijersbergen, A. G. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol. 16(9):839-842.

Dürrenberger, F, Crameri, A., Hohn, B., and Koukolikova-Nicola., Z. 1989. Covalently bound VirD2 of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc. Natl. Acad. Sci. U.S.A. 86(23):9154-9158.

Gelvin, S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:223-256.

Gelvin, S.B. 2010. Finding a way to the nucleus. Curr. Opin. Microbiol. 13(1):53-58.

Guo, M., Hou, Q., Hew, C.L., and Pan, S.Q. 2007a. *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. Mol Plant-Microbe Int. 20(10):1201-1212.

Guo, M., Jin, S., Sun, D., Hew, C.L., and Pan, S.Q. 2007b. Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type IV secretion apparatus. Proc. Natl. Acad. Sci. U.S.A. 104(50):20019-20024.

Hooykaas, P.J.J., Hofker, M., Den Dulk-Ras, H., and Schilperoort, R.A. 1984. A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. Plasmid. 11(3):195-205.

Hooykaas-van Slogteren, G. M., Hooykaas, P. J., and Schilperoort, R. A. 1992. Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens*. Biotechnology. 24:382-383.

Howard, E. A., Zupan, J. R., Citovsky, V., and Zambryski, P. C. 1992. The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localisation signal: implications for nuclear uptake

of DNA in plant cells. Cell. 68(1):109-118.

Jayaswal, R. K., Veluthambi, K., Gelvin, S. B., and Slightom., J. L. 1987. Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a *virD*-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J. Bacteriol. 169(11):5035-5045.

Judd, P.K., Kumar, R.B., and Das. A. 2005. Spatial location and requirements for the assembly of the *Agrobacterium tumefaciens* type IV secretion apparatus. Proc. Natl. Acad. Sci. U.S.A. 102(32):11489-11503.

Koekman, B. P., Hooykaas, P. J. J. and. Schilperoort R. A. 1982. Localization of the replication control region on the physical map of the octopine Ti plasmid. Plasmid. 7(2):119-132.

Kumar, R.B., and Das, A. 2002. Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein VirD4. Mol. Microbiol. 43(6):1523-1532.

Lanka, E., and Wilkins, B.M. 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. 64:141-169.

Lessl, M., and Lanka, E. 1994. Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell. 77(3):321-324.

Llosa, M., Gomis-Ruth, F.X., Coll, M., and de la Cruz, F. 2002. Bacterial conjugation: a two-step mechanism for DNA transport. Mol. Microbiol. 45(1):1-8.

Mysore, K. S., Bassuner, B., Deng, X. B., Darbinian, N. S., Motchoulski, A., Ream, W., and Gelvin, S. B. 1998. Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. Mol. Plant-Microbe Interact. 11(7):668-683.

Pitzschke, A, and Hirt, H. 2010. New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. EMBO (Eur. Mol. Biol. Organ.) J. 29(6):1021-1032.

Rossi, L., Hohn, B., and Tinland, B. 1993. The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. Mol. Gen. Genet. 239(3):345-353.

Scheiffele, P., Pansegrau, W., and Lanka, E. 1995. Initiation of *Agrobacterium tumefaciens* T-DNA processing. Purified proteins VirD1 and VirD2 catalyze site- and trans-specific cleavage of superhelical T-border DNA *in vitro*. J. Biol. Chem. 270(3):1269-1276.

Schrammeijer, B., den Dulk-Ras, A., Vergunst, A.C., Juralda Jácome, E., and Hooykaas, P.J.J. 2003. Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. Nucleic Acids Res. 31(3):860-868.

Schulein, R., Guye, P., Rhomberg, T.A., Schmid, M.C., Schröder, G., Vergunst, A.C., Carena, I., and Dehio, C. 2005. A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. Proc. Natl. Acad. Sci. U.S.A. 102(3):856-861.

Shurvinton, C. E., Hodges, L., and Ream, W. 1992. A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. Proc. Natl. Acad. Sci. U.S.A. 89(24):11837-11841.

Steck, T.R., Lin, T.S., and Kado, C.I. 1990. VirD2 gene product from the nopaline plasmid pTiC58

has at least two activities required for virulence. Nucleic Acids Res. 18(23):6953-6958.

Tzfira, T., Vaidya, M., and Citovsky, V. 2004. Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature. 431(7004):87-92.

Van Kregten, M, Lindhout, B.I., Hooykaas, P.J.J, and van der Zaal, B.J. 2009. *Agrobacterium*-mediated T-DNA transfer and integration by minimal VirD2 consisting of the relaxase domain and a Type IV Secretion System translocation signal. Mol. Plant-Microbe Interact. 22(11): 1356-1365.

Vergunst, A.C., van Lier, M.C., den Dulk-Ras, A., Stuve, T.A., Ouwehand, A., and Hooykaas, P.J.J. 2005. Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. Proc. Natl. Acad. Sci. U.S.A. 102(3):832-837.

Vergunst, A.C., Schrammeijer, B., den Dulk-Ras, A., de Vlaam, C.M., Regensburg-Tuïnk, T.J., and Hooykaas, P.J.J. 2000. VirB/D4-dependent protein translocation from *Agrobacterium tumefaciens* into plant cells. Science. 290(5493):979-982.

Vogel, A.M., and Das, A. 1992. Mutational analysis of *Agrobacterium tumefaciens virD2*: tyrosine 29 is essential for endonuclease activity. J. Bacteriol. 174(1):303-308.

Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M., and Chilton, M.D. 1982. Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proc. Natl. Acad. Sci. U.S.A. 79(20):6322-6326.

Zhu, J., Oger, P.M., Schrammeijer, B., Hooykaas, P.J.J., Farrand, S.K., and Winans, S.C. 2000. The bases of crown gall tumorigenesis. J. Bacteriol. 182(14):3885-3895.