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## VirD2 of *Agrobacterium tumefaciens* : functional domains and biotechnological applications

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### Citation

Kregten, M. van. (2011, May 19). *VirD2 of Agrobacterium tumefaciens : functional domains and biotechnological applications*. Retrieved from <https://hdl.handle.net/1887/17648>

Version: Corrected Publisher's Version

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**Note:** To cite this publication please use the final published version (if applicable).

# Chapter 2

## ***Agrobacterium*-Mediated T-DNA Transfer and Integration by Minimal VirD2 Consisting of the Relaxase Domain and a Type IV Secretion System Translocation Signal**

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Modified from Van Kregten *et al.*, 2009,  
Mol. Plant-Microbe Interact. 22(11):1356-1365.

## Abstract

The VirD2 protein of *Agrobacterium tumefaciens* is essential for processing and transport of the T-DNA. It has at least three functional domains: a relaxase domain at the N-terminus, a bipartite nuclear localization signal (NLS), and a sequence called  $\omega$  at the C terminus. We confirm here that deletions of the C-terminal part of VirD2 led to lack of transfer of T-DNA but, for the first time, we report that virulence is restored when these truncations are supplemented at the C terminus by a short translocation signal from the VirF protein. The lack of virulence of C-terminal deletions suggests that the C-terminal part contains all or part of the translocation signal of VirD2. Using a novel series of mutant VirD2 proteins, the C-terminal half of VirD2 was further investigated. We demonstrate that the C-terminal 40 amino acids of VirD2, which include the NLS and  $\omega$ , contain all or part of the translocation domain necessary for transport of VirD2 into plant cells, while another element is present in the middle of the protein. The finding that a type IV secretion system transport signal at the C-terminus of VirD2 is necessary for virulence provides evidence for the role of VirD2 as the protein driving translocation of the T-strand from *Agrobacterium tumefaciens* into plant cells.

## Introduction

Throughout recent decades, *Agrobacterium tumefaciens* has been widely used as a tool for the genetic engineering of a variety of organisms, which include dicotyledonous plants, monocotyledonous plants (Hooykaas-van Slogteren *et al.*, 1984), yeast (Bundock *et al.*, 1995), and other fungi (de Groot *et al.*, 1998).

During transformation by *A. tumefaciens*, a single-stranded (ss)DNA segment is transferred from the bacterium to the recipient cell, where it is integrated into the genome. This transferred segment or T-strand, also known as T-DNA, is derived from a particular region (T-region) of a tumor-inducing (Ti)-plasmid, where it is flanked by two imperfect direct repeats, the left (LB) and right (RB) border sequences. Among the Ti plasmid-encoded virulence (Vir) proteins that are required for T-strand formation and translocation to the recipient cell (Christie and Vogel 2000, Zhu *et al.*, 2000), VirD2 is absolutely

essential. Together with VirD1, VirD2 forms a relaxosome at the RB of the T-region, which initiates the formation of the T-strand. VirD1 and VirD2 are both required for recognition of the border repeat sequences flanking the T-strand but it is the relaxase domain of the VirD2 protein which actually cleaves the LB and RB sequence on one of the DNA strands, enabling the release of the T-strand (Jayaswal *et al.*, 1987, Lessl and Lanka 1994, Scheiffele *et al.*, 1995). VirD2 remains covalently attached to the 5' end of the T-strand through an N-terminal tyrosine residue (Tyr29) (Dürrenberger *et al.*, 1989, Scheiffele *et al.*, 1995, Vogel and Das 1992).

After its formation within *A. tumefaciens*, the VirD2-Tstrand complex is transported to the cytoplasm of the recipient eukaryote, along with but independently from other virulence proteins such as VirE2, VirF (Vergunst *et al.*, 2000), VirD5 (Vergunst *et al.*, 2005), and VirE3 (Schrammeijer *et al.*, 2003). These transport or translocation events are mediated by the type IV secretion system (T4SS) of *A. tumefaciens*, a product of the *virB* operon, associated with the coupling protein VirD4 (Christie *et al.*, 2005). It is a matter of debate how the complex is translocated into recipient cells, whether translocation is driven by the relaxase or by the T-DNA. To support the favored model of relaxase-driven translocation of the T-DNA (Cascales and Christie 2003, Chen *et al.*, 2005), evidence has been found of low levels of translocation of relaxases MobA (plasmid RSF1010) and VirD2 without the presence of T-DNA (Vergunst *et al.*, 2005), indicating that transit through the T4SS is possible for unbound relaxases. However, the fact that translocation of these proteins is only detected at a very low level could very well mean that the presence of a covalently bound T-DNA moiety is the normal prerequisite for their transfer. In that manner, recognition and transfer of T-DNA by the T4SS rather than the relaxase would be the predominant reason for the transport of relaxases. This issue is still not resolved. A possible mechanism for T-DNA-driven translocation has been suggested when it was discovered that TrwB, the coupling protein of the T4SS of plasmid R388, possesses DNA-dependent ATPase activity (Tato *et al.*, 2005). Another model suggests that ssDNA binding VirE2 proteins in the recipient cell form a structure that can pull the T-strand into the recipient cell (Grange *et al.*, 2008).

It is hypothesized that VirE2 binds to the T-strand in the cytoplasm of the recipient cell and aids in protection of the T-strand from attack by nucleases (Citovsky *et al.*, 1989). VirD2 is thought to do the same for the 5' end (Dürrenberger *et al.*, 1989). Although VirD2 and VirE2 both contain one or more nuclear localization signals (NLS), nuclear targeting of the T-strand complex has been primarily attributed to an NLS of VirD2. Although the

N-terminal part of VirD2 proteins contains a monopartite NLS composed of amino acids 32 through 35 (KRAR) (Rossi *et al.*, 1993), the very C-terminal end of VirD2, which is conserved between different VirD2 proteins, has a bipartite NLS. Both of them are functional in a modular fashion, able to guide reporter proteins into the nucleus, but the C-terminal bipartite NLS has been shown to be essential for nuclear uptake of the T-complex (Howard *et al.*, 1992, Relić *et al.*, 1998, Tinland *et al.*, 1995). Recently, it was shown that VirE2 is able to interact with importin  $\alpha$  when bound to ssDNA, suggesting a role for both VirD2 and VirE2 in nuclear uptake of the T-complex (Bhattacharjee *et al.*, 2008).

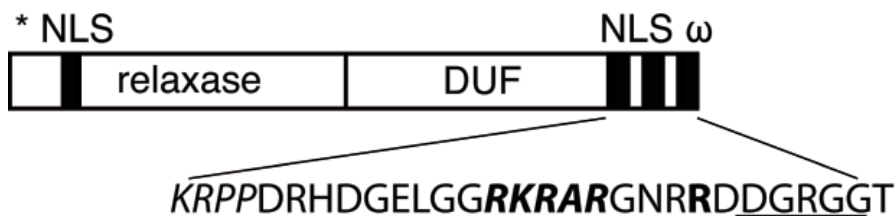
The VirD2 protein encoded by the Ti-plasmid of the widely used octopine type strains of *Agrobacterium* is a 47.5-kDa protein of 424 amino acids (Fig. 1). Alignment of VirD2 proteins from different *Agrobacterium* strains has shown that the protein consists of two distinct parts. Although the N-terminal relaxase domains are virtually identical, the C-terminal halves of VirD2 proteins are rather variable (Howard *et al.*, 1992, Shurvinton *et al.*, 1992). Despite this variation, several conserved sequences are present at the C-terminal end of VirD2. Howard and associates (1992) aligned VirD2 proteins from different *Agrobacterium* strains and found that the bipartite NLS is conserved at the C-terminal end. Shurvinton and associates (1992) also aligned the C-terminal end of different VirD2 proteins and identified the bipartite NLS and the  $\omega$  sequence as conserved between the different VirD2 proteins.

Based on an alignment of the C terminus of translocated proteins (including VirD2, VirF, VirE2, VirE3, and the relaxase MobA of plasmid RSF1010), a putative C-terminal translocation signal containing several conserved arginine residues was annotated (Vergunst *et al.*, 2000) (Fig. 1). In VirD2, this signal partly overlaps with the bipartite NLS also present at the C-terminal end.

The very C-terminal amino acid sequence, designated the omega ( $\omega$ ) sequence (DGRGG) (Fig. 1), has been shown to be important for the function of VirD2. Insertion or deletion of amino acids of  $\omega$  was shown to have detrimental effects on the final number of T-strand integration events in recipient plant cell genomes (Bravo-Angel *et al.*, 1998, Shurvinton *et al.*, 1992). However, the precise role of VirD2 regarding T-strand integration events has been the object of debate. Mysore and associates (1998) reported  $\omega$  to be important for integration of the T-DNA into the host genome. However, Tinland and associates (1995) reported that this might at least partially be a consequence of VirD2-mediated protection of the 5' end of the T-strand against nucleolytic attack, thus not necessarily reflecting a reduced efficiency of the integration step itself. Furthermore, Bravo-Angel and associates (1998)

published that  $\omega$  was important for T-DNA translocation to the recipient cell, whereas the efficiency and the pattern of integrations were unaffected by  $\omega$  mutations.

VirD2 contains a large domain of unknown function (DUF) between the relaxase domain and the very C-terminal end (Fig. 1). This DUF consists of amino acids residues which are poorly conserved between different VirD2 proteins (Howard *et al.*, 1992). Although substantial in size, no enzymatic functions have thus far been attributed to the VirD2 DUF. However, it would be surprising if rapidly evolving bacteria would maintain such a large domain when it had no function. If it has no enzymatic activity, it might be required for recruiting interaction partners in the recipient cell. Indeed, several *Arabidopsis thaliana* cyclophilins were shown to interact with sequences within the DUF (Deng *et al.*, 1998).



**Figure 1:** Schematic representation of the VirD2 coding region. Within the N-terminal 228 amino acids, the relaxase domain is encoded, which is essential for T-strand processing (Scheiffele *et al.*, 1996; Steck *et al.*, 1990). Tyrosine residue 29 (indicated by \*) binds covalently to the 5' end of the T-strand (Vogel *et al.*, 1992). The N-terminal nuclear localization signal (NLS) (Rossi *et al.*, 1993) is indicated by a black box. The domain of unknown function (DUF) of VirD2 consists mainly of amino acid residues which are poorly conserved between VirD2 proteins (Howard *et al.*, 1992). At the very C-terminal end, which consists of approximately 40 amino acids that are conserved between different VirD2 proteins, the bipartite NLS (in italics) has been shown to function in nuclear uptake of the T-complex (Relić *et al.*, 1998; Tinland *et al.*, 1995). The  $\omega$  domain (in italics and underlined), another element of the conserved very C-terminal end, has been described as being important for T-DNA transfer (Bravo-Angel *et al.*, 1998) or for integration of T-DNA within the plant genome (Mysore *et al.*, 1998; Shurvinton *et al.*, 1992). The C-terminal 40 amino acids of VirD2 contain a consensus sequence implicated in translocation through the type IV secretion system of *Agrobacterium tumefaciens* (indicated in bold) (Vergunst *et al.*, 2005). Figure not drawn to scale.

In this study, we used a novel series of mutants of VirD2 to investigate the requirements for translocation and integration of T-strands into recipient plant cells. Evidence is presented that part of the DUF and the C-terminal 40 amino acids are required for translocation of the VirD2-T-strand complex but that the interactions of plant proteins

with the DUF described thus far do not influence the transformation efficiency. A minimal version of VirD2, consisting of only the relaxase domain, still meets the requirements for translocation when supplemented with a short translocation signal derived from VirF. The *virF* gene is present only in some Ti plasmids. Apart from its ability to enhance the virulence of octopine strains of *Agrobacterium* on some host plants, the native VirF protein is not at all involved in T-strand formation or its transfer to host cells (Regensburg-Tuïnk and Hooykaas 1993). Altogether, the data reported here provide compelling evidence that, once a T-strand has been made within *A. tumefaciens*, the translocation of the VirD2-T-strand complex is protein driven rather than DNA driven. Moreover, once inside a recipient plant cell, the DUF is not required for transformation of the recipient cell. The broader significance of these results is discussed.

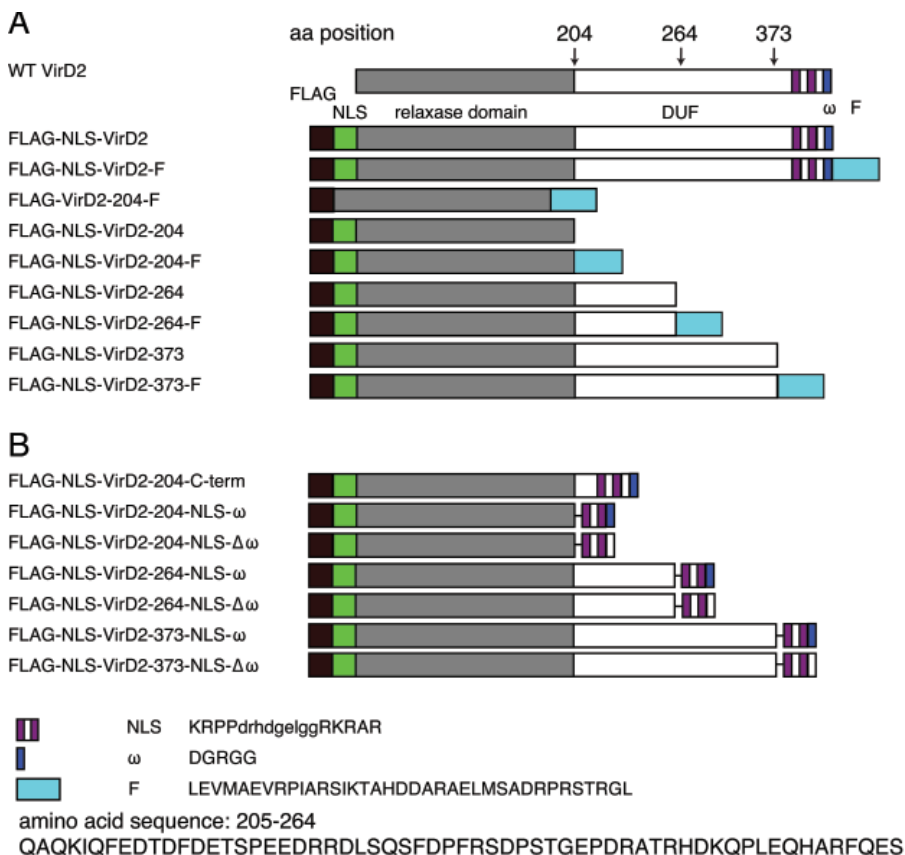
## Results

### **Providing VirD2 with FLAG and SV40 NLS sequences and the VirF protein translocation signal.**

In our study, we investigated in detail which sequences within the C-terminal DUF of the VirD2 protein are essential for virulence of *A. tumefaciens* and for translocation of T-strands to plant cells. For this purpose, we made a set of VirD2 C-terminal deletion constructs. For convenience of protein immunodetection, we added a FLAG tag at the N terminus. To ensure nuclear targeting when the endogenous C-terminal bipartite NLS of VirD2 was deleted, an SV40 NLS was added downstream of the FLAG tag, N-terminal of the VirD2 moieties (Fig. 2). In order to compensate for the possible loss of a T4SS secretion signal, which was postulated to be present at the very C-terminal end (Vergunst *et al.*, 2005), we also made a series of constructs to which we added the C-terminal 37 amino acids of VirF (F). This particular F sequence was shown to enable translocation of proteins through the T4SS of *A. tumefaciens* using Cre-fusion proteins to detect protein translocation directly (Vergunst *et al.*, 2005).

Prior to testing VirD2 deletion constructs, we first tested the transfer of T-DNA via the tagged VirD2 derivatives that formed the starting point of the experiment. Hence, the activities of FLAG-NLS-VirD2 and FLAG-NLS-VirD2-F were established in *Arabidopsis* root transformation assays, using *Agrobacterium* sp. strain LBA2585 ( $\Delta virD2$  and  $\Delta T$ -DNA).

The wide-host-range plasmid pCAMBIA2301, which contains a T-DNA sequence with the plant-selectable *nptII* gene and a  $\beta$ -glucuronidase (*GUS*):*intron* reporter gene between the T-DNA border repeats, was used as a T-strand donor. In such a root transformation assay, GUS staining directly after the 3 days of cocultivation can be taken as a measure for the total amount of T-strand molecules that have been transferred from the bacterial cell to the nuclei of plant cells; thus, those that have already been integrated into the genome as well as those that have not yet been incorporated and might be only transiently present (Tinland *et al.*, 1995).



**Fig. 2. A**, Schematic overview of the protein structure of VirD2 truncations. **B**, Schematic overview of the protein structure of VirD2 truncations complemented with the different elements of the C-terminal end. Pictures not drawn to scale.



The root transformation assays (Table 1) clearly demonstrated that fusing a FLAG-NLS sequence at the N terminus of VirD2 did not abolish its activity, although it reduced the transformation efficiency by approximately 60% when compared with wild-type (WT) VirD2 protein. Therefore, the VirD2 protein tolerates the N-terminal modifications while retaining most of its T-strand transferring capacity. The C-terminal addition of F did not hamper VirD2 function at all. In fact, FLAG-NLS-VirD2-F is transferred at the efficiency of WT VirD2. However, C-terminal addition of F to WT VirD2, without FLAG and NLS, resulted in a twofold reduction of transformation rate (data not shown), indicating that WT levels of transformation cannot be boosted further by the F sequence

**Table 1.** Root transformation assays

<b>Construct<sup>a</sup></b>	<b>Transient transformation<sup>b</sup></b>
WT VirD2	1
FLAG-NLS-VirD2	0.39 ± 0.13
FLAG-NLS-VirD2-F	1.07 ± 0.21
FLAG-VirD2-F	0.89 ± 0.20

<sup>a</sup> WT = wild type, NLS = nuclear localization signal.

<sup>b</sup> For every experiment, at least 100 explants were scored for β-glucuronidase-positive spots. Data were normalized against data for WT VirD2. n = 3.

### **Deletion of the DUF of FLAG-NLS-VirD2 still allows proficient transient and stable T-DNA transfer to *A. thaliana* root explants when F is present.**

We investigated the effects of deletions in the C terminus of FLAG-NLS-VirD2 while leaving the N-terminal relaxase domain intact, because relaxase activity is obviously essential for T-DNA processing (Lessl *et al.*, 1994, Scheiffele *et al.*, 1995). Although the smallest version of VirD2 that was still able to process DNA correctly was previously reported to consist of 228 amino acids (Scheiffele *et al.*, 1995, Steck *et al.*, 1990), we decided to further minimize the relaxase domain of VirD2 to 204 amino acids, based upon sequence similarities between different relaxases.

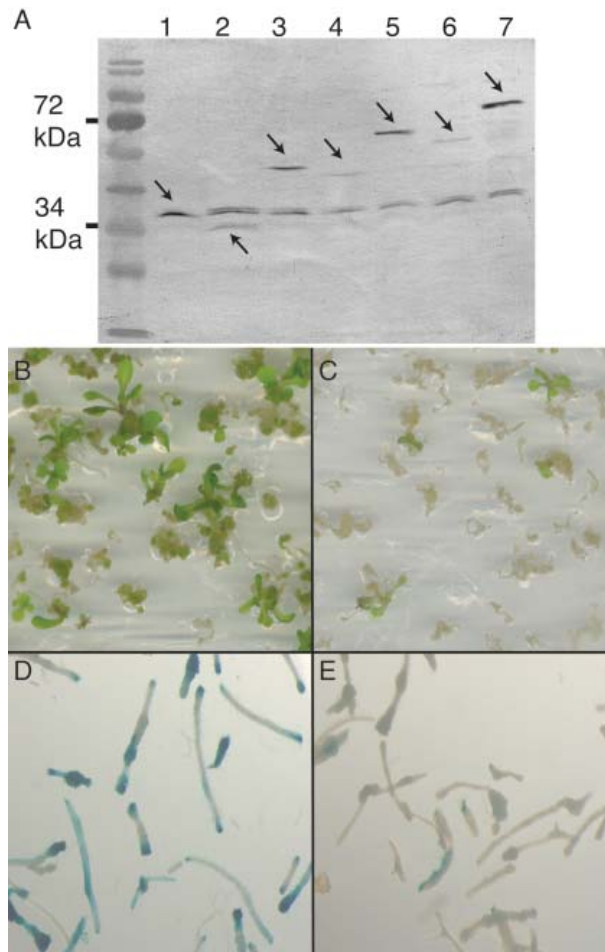
The first series of deletion mutants (Fig. 2) comprised the following proteins: FLAG-NLS-VirD2-204, consisting of the N-terminal 204 amino acids of VirD2, which is the relaxase domain; FLAG-NLS-VirD2-264, consisting of the N-terminal 264 amino acids of VirD2, which is the relaxase domain and part of the DUF; FLAG-NLS-VirD2-373, consisting of VirD2, from which only the very C-terminal 51 amino acids have been

truncated, which includes the 40 amino acids in which the translocation signal is most likely present.

The expression of the various chimerical proteins in acetosyringone- induced *Agrobacterium* strains was determined by Western blotting using an anti-FLAG antibody (Fig. 3A). In all samples, a clear signal was present at a somewhat higher-than expected position but agreeing well with predicted molecular weights. Fusion with F adds 4 kDa to the protein. Degradation seemed to be minimal, suggesting that the different FLAG -NLS-VirD2 truncations were expressed and were stable in *A. tumefaciens*. Proteins containing a C-terminal F translocation signal seemed to be produced in higher amounts than those without F.

In order to evaluate the plant-cell-transforming ability of the different *Agrobacterium* strains, it was decided to use root explants of *A. thaliana*. In this assay, even low amounts of transformation events are easily detected. GUS staining directly after cocultivation was taken as a measure for transient transformation, and the number of successful integrations was measured by the number of transgenic kanamycin-resistant calli that developed after several weeks on selective medium. All data were normalized against WT VirD2 (VirD2 expressed under the control of the *virD* promoter).

Translocation of T-strands by the FLAG-NLS-VirD2 C-terminal truncations was completely abolished (Table 2). Not even FLAG-NLS-VirD2-373, which is only deleted for the C-terminal 51 amino acids, was able to induce any GUS activity or callus formation, indicating that the very C-terminal end provides an essential function in translocation of the VirD2-Tcomplex. However, by providing a C-terminal VirF (F) translocation sequence to the FLAG-NLS-VirD2 truncations, translocation of T-strands was to a large extent restored. This effect was not enhanced by the presence of longer parts of the DUF (Table 2). The F sequence apparently was sufficient as a dominant modular domain enabling the passage of hybrid proteins and their covalently bound T-strands into recipient plant cells. No obvious difference between the ratio of transient transformation and stable integration was observed for any of the constructs, indicating that the DUF and the C-terminal end of VirD2 have no special role in T-DNA integration efficiency. To determine the necessity of the addition of the SV40 NLS, we created FLAG-VirD2-204-F, in which no extra NLS is present. Surprisingly, it performed at an even higher level than FLAG-NLS-VirD2-204-F, indicating that either the small NLS present in the relaxase domain (Rossi *et al.*, 1993) is sufficient for nuclear localization or that VirD2 is guided to the nucleus by another protein or by the VirF protein translocation signal. However, no previously undescribed putative NLS sequences



**Fig. 3. A**, Expression of VirD2 truncation mutants. Western blot showing expression of FLAG-NLS-VirD2-(F) and truncations thereof. Arrows indicate position of the bands corresponding to the FLAG-NLS-VirD2 truncations. Left to right: FLAG-NLS-VirD2-204-F, FLAG-NLS-VirD2-204, FLAG-NLS-VirD2-264-F, FLAG-NLS-VirD2-264, FLAG-NLS-VirD2-373-F, FLAG-NLS-VirD2-373, and FLAG-NLS-VirD2-F. In all lanes, a nonspecific band is also present. **B** and **C**, Callus formation after 3 weeks in *Arabidopsis thaliana* root explants transformed with *Agrobacterium tumefaciens* LBA2585 carrying pCAMBIA2301 and expressing **B**, wild-type (WT) VirD2 and **C**, FLAG-NLS-VirD2-264-NLS- $\Delta\omega$ . **D** and **E**,  $\beta$ -Glucuronidase-positive spots in *Arabidopsis thaliana* root explants transformed with *Agrobacterium tumefaciens* LBA2585 carrying pCAMBIA2301 and expressing **D**, WT VirD2 and **E**, FLAG-NLSVirD2-264-NLS- $\Delta\omega$ .

could be identified in VirF (PredictNLS program.)

### **Domains of VirD2 required for translocation**

Considering the experiments described above, the complete DUF as well as the remaining C-terminal sequences of VirD2 can, in fact, be replaced with a short VirF-derived protein translocation sequence which, by itself, is unlikely to perform any functions other than mediating passage through the T4SS. At the same time, because none of the C-terminal VirD2 deletion constructs were competent for translocation without F, part of the native VirD2 C terminus must be required for translocation. It has been reported that the native VirD2 protein contains a T4SS translocation sequence of its own and a candidate sequence at the C-terminal end has been postulated (Vergunst *et al.*, 2005). In order to define the sequences of VirD2 that are sufficient for translocation, we created internal deletion mutations in the DUF. Between the relaxase domain and the re-added C-terminal sequences, a linker sequence encoding the amino acids GGGs was provided to ensure flexibility between the two linked sequences (Wriggers *et al.*, 2005). We thus created FLAG-NLS-VirD2-204-C-term, consisting of the relaxase domain and the C-terminal sequence downstream from amino acid 374 (51 amino acids). This sequence includes the putative translocation signal, including the bipartite NLS and the  $\omega$  domain (Fig. 2). To address the question of which C-terminal sequences are required for translocation, we also created FLAG-NLS-VirD2-204-NLS- $\omega$ , consisting of the relaxase domain, the C-terminal bipartite NLS, and  $\omega$  (Fig. 2). In this construct, a 4-amino-acid sequence (GNRR) is deleted between the bipartite NLS and  $\omega$ . We also created FLAG-NLS-VirD2-204-C-term- $\Delta\omega$ , consisting of the C-terminal sequence from amino acids 396 through 418. This sequence includes the bipartite NLS and the arginine-rich region but has  $\omega$  precisely deleted. Using these VirD2 truncations, we tested for the effects of a full DUF deletion on competence of the truncated VirD2 protein for T-DNA translocation and virulence of the corresponding *Agrobacterium tumefaciens* strain. As was already evident from the earlier series of experiments (Table 2), a FLAG-NLS-VirD2-204 version of VirD2 without any further C-terminal additions was completely unable to deliver T-strands into plant cells. By adding any of the short VirD2-derived C-terminal sequences mentioned above, it was reproducibly found that the resulting FLAG-NLS-VirD2-204 derivatives were all able to deliver T-strands to *Arabidopsis* root explants, albeit only at a 1 to 5% level of the WT VirD2 protein (Table 3). Hence, for

restoration of appreciable VirD2 activity with VirD2-derived C-terminal sequences, the presence of at least part of the DUF seemed to be required. Therefore, FLAG-NLS-VirD2-264-NLS- $\omega$  and FLAG-NLS-VirD2-264- C-term- $\Delta\omega$  were created, as well as the full DUF-containing versions FLAG-NLS-VirD2-373-NLS- $\omega$  and FLAG-NLSVirD2- 373-C-term- $\Delta\omega$ . Remarkably, all these proteins could restore the virulence of VirD2 to the level conferred by FLAG-NLS- VirD2.

Altogether, our results show that the very C-terminal sequence of VirD2 does not contain a translocation signal that is as efficient as F in supporting T-DNA translocation. Furthermore, in our work, we did not find a reduction in T-DNA translocation when  $\omega$  was deleted or a small internal deletion of 4 amino acids was present between NLS and  $\omega$ . However, when VirD2-derived C-terminal sequences were used instead of the F, the presence of a small internal part of the DUF corresponding to amino acids 205 to 264 of WT VirD2 restored the virulence of the corresponding *A. tumefaciens* strains to the same level as the ones that possessed F as a C-terminal sequence. Addition of the larger part of DUF (amino acids 205 to 373) did not lead to any further enhanced T-strand transfer, thus providing compelling evidence that the sequence of DUF between amino acids 205 and 264 confers an important function in T-DNA translocation within the native VirD2 protein.

**Table 2:** C-terminal truncations

Construct <sup>a</sup>	Transient transformation <sup>b</sup>	Stable integration <sup>c</sup>
WT VirD2	1	1
FLAG-NLS-VirD2-F	1.07 ± 0.21	0.92 ± 0.17
FLAG-VirD2-204-F	0.82 ± 0.13	nd
FLAG-NLS-VirD2-204	0	0
FLAG-NLS-VirD2-204-F	0.57 ± 0.19	0.51 ± 0.21
FLAG-NLS-VirD2-264	0	0
FLAG-NLS-VirD2-264-F	0.61 ± 0.21	0.49 ± 0.23
FLAG-NLS-VirD2-373	0	0
FLAG-NLS-VirD2-373-F	0.65 ± 0.21	0.61 ± 0.22

<sup>a</sup>WT = wild type, NLS = nuclear localization signal. <sup>b</sup>For every experiment, at least 100 explants were scored for  $\beta$ -glucuronidase-positive spots. Data were normalized against data for WT VirD2. <sup>c</sup>Amount of calli per explants was scored 3 and 4 weeks after cocultivation. Data were normalized against WT VirD2. For every experiment, at least 100 explants were scored for callus formation. nd = not determined. *n* = 3.

**Table 3.** Root transformation assays

<b>Construct<sup>a</sup></b>	<b>Transient transformation<sup>b</sup></b>	<b>Stable integration<sup>c</sup></b>
WT VirD2	1	1
FLAG-NLS-VirD2	0.39 ± 0.13	n.d.
FLAG-NLS-VirD2-204	0	0
FLAG-NLS-VirD2-204-C-term	0.05 ± 0.01	0.04 ± 0.01
FLAG-NLS-VirD2-204-C-term-Δω	0.05 ± 0.01	0.01 ± 0.01
FLAG-NLS-VirD2-204-NLS-ω	0.08 ± 0.01	n.d.
FLAG-NLS-VirD2-264-NLS-ω	0.33 ± 0.19	0.25 ± 0.15
FLAG-NLS-VirD2-264-C-term-Δω	0.41 ± 0.13	0.22 ± 0.10
FLAG-NLS-VirD2-373-NLS-ω	0.37 ± 0.01	0.24 ± 0.04
FLAG-NLS-VirD2-373-C-term-Δω	0.40 ± 0.18	n.d.

a WT = wild type, NLS = nuclear localization signal. b For every experiment, at least 100 explants were scored for β-glucuronidase- positive spots. Data were normalized against data for WT VirD2. c Amount of calli per explants was scored 3 and 4 weeks after cocultivation. Data were normalized against WT VirD2. For every experiment, at least 100 explants were scored for callus formation. n.d. = not determined

## Discussion

By constructing a novel series of VirD2 mutants which was used for *Arabidopsis* and *Nicotiana glauca* transformations, we have shown that the large C-terminal half of VirD2, mostly consisting of a large DUF, can, in fact, be deleted without any serious consequences for the normal VirD2 functions, provided that a short 37-amino-acid translocation signal (F) of the VirF protein is present instead. These data shed a new light upon the truly essential domains of VirD2 in members of genus *Agrobacterium* itself and for the translocation of VirD2 via the T4SS of *A. tumefaciens*, a paradigm for conjugative DNA transfer and effector protein translocation.

Although multiple protein substrates have now been identified for the T4SS of *A. tumefaciens* (e.g., VirE2, VirE3, and VirF) (Citovsky *et al.*, 1989, Christie 2004, García-Rodríguez *et al.*, 2006), definitive proof that T-strand transfer is guided by the VirD2 protein is, in fact, lacking. For TrwC, the relaxase and helicase of plasmid R388, translocation independent of DNA transfer to the recipient has been shown (Draper *et al.*, 2005), although its efficacy remained unknown. Regarding VirD2, Cre recombinase reporter assays for protein translocation (CRAFT) assays indicated that VirD2 can mediate

Cre transfer to plant cells in the absence of T-DNA, albeit at a very low level (Vergunst *et al.*, 2005). For our experiments, CRAfT assays based upon the recombinogenic activity of Cre within plant cells were not feasible. Fusion of VirD2 to the N or C terminus of Cre resulted in near complete loss of recombination events in planta (*unpublished data*). We have taken these data as indications that either the Cre protein does not tolerate a VirD2 moiety or such fusions can no longer be translocated. Using T-strand transfer as a reporter system, as was done in our study, we present an alternative to CRAfT which, moreover, could give insights regarding the actual function of VirD2 in T-strand formation and translocation. Our data clearly demonstrated that properties of the VirD2 relaxase protein are the main determinants behind T-DNA translocation from *A. tumefaciens* through the T4SS into plant cells. Once delivered to the T4SS, DNA-dependent ATPase activity of coupling proteins can subsequently generate the energy required for DNA transfer (Llosa *et al.*, 2002, Tato *et al.*, 2005), or perhaps VirE2 can aid the translocation of the T-DNA from the cytoplasm of the recipient cell (Grange *et al.*, 2008).

The conclusion that T-strand translocation first of all depends on translocation of the VirD2 relaxase and not, by any means, upon the transferred DNA itself was particularly evident for the following reason: all VirD2 truncations based upon FLAG-NLS-tagged VirD2, including a complete truncation of the DUF, performed at 50 to 60% of WT VirD2 in a root transformation assay, provided that they were equipped with a C-terminal translocation signal of VirF (F). In none of the cases was any obvious difference between transient transformation (GUS staining directly after cocultivation) and stable integration (formation of kanamycin resistant calli) observed. Removal of F abolished T-strand translocation completely, strongly suggesting that C-terminal sequences of VirD2 are, indeed, required for translocation, thus corroborating data from other types of VirD2 mutants (Bravo-Angel *et al.*, 1998, Mysore *et al.*, 1998, Shurvinton *et al.*, 1992).

Our finding that DUF deletion mutants could still function in translocation of T-strands confirmed and extended earlier data that part of the DUF is not essential for VirD2 function (Koukolíková-Nicola *et al.*, 1993). In that study, it was shown that 111 to 142 amino acids of the C-terminal end of VirD2 could be deleted, provided that a 38-amino-acid VirD2 C-terminal sequence or the larger part thereof was fused to the N-terminal 275 amino acids. Although these data suggested that the C-terminal end of VirD2 could function as a rather independent moiety that was able to complement a severe C-terminal truncation, our data demonstrate that the situation is more complex. When the DUF extends to amino acid 373 or 264 of VirD2, it is, indeed, possible to complement VirD2 function with a short

VirD2-derived C-terminal sequence (Table 3). However, further truncation of the DUF down to amino acid residue 204 yielded an N-terminal half of VirD2 that was refractory to complementation with VirD2-derived C-terminal sequences but the protein regained T-strand delivering capacity when supplemented with F. Our data provide the first evidence that the amino acid sequence flanked by residues 204 and 264 has a biological function, in contrast to the larger C-terminal part of the DUF. This amino acid sequence does not show similarity to any proteins other than VirD2 and contains no known domains, as determined by BLAST, Pfam, and Scan- Prosite searches. As will be discussed below, the 204-to-264 sequence is most likely important for translocation processes within *A. tumefaciens*. On the other hand, our data strongly indicate that the 37-amino-acid VirF translocation signal is extremely potent, possibly combining recruitment to the T4SS within *A. tumefaciens* cells as well as passage through the T4SS to eukaryotic cells.

As far as reported interactions of VirD2 domains with host-encoded proteins are concerned, our data, in combination with the earlier VirD2 truncations (Koukolíková-Nicola *et al.*, 1993), enabled a reevaluation regarding their biological relevance. Some plant cyclophilins - proteins involved in protein folding, protein degradation, and signal transduction (Wang and Heitman 2005) - have been reported to interact with VirD2 (Deng *et al.*, 1998). Roc1 was found to interact with amino acids 174 to 337 of VirD2. The full interaction domain with Roc1 is only present in our longest VirD2 truncation, VirD2-373. CypA was reported to interact with amino acids 274 to 337 of VirD2. Only FLAG-NLS-VirD2-373-F contains this interacting domain. Because, in our experiments, plant cell transformation via these truncated VirD2 proteins was easily obtained, these cyclophilins can be of only limited importance for VirD2 function *in planta*. Of course, it cannot be excluded that these interactions are necessary for correct folding of WT VirD2 but they apparently are not required for folding of the deletion constructs. Moreover, a study in our lab showed that deletion of all cyclophilins in a yeast strain did not inhibit transformation by *A. tumefaciens* (H. Van Attikum and P. J. J. Hooykaas, *unpublished results*). A biologically relevant interaction with the TATA box-binding protein, which binds to the N-terminal half of VirD2 (Bakó *et al.*, 2003), is still possible in our mutants.

Based upon our series of constructs, the 51-amino-acid sequence forming the very C terminus of VirD2 does not contain any elements sufficient for mediating efficient T-strand transfer to *Arabidopsis* root cells. Obviously, when transfer of T-strands to host cells is inhibited, their integration into the host genome will not be observed. Mostly for this reason, the involvement of VirD2-derived sequences during T-DNA integration into



the host genome has, thus far, been controversial (Bravo-Angel *et al.*, 1998, Mysore *et al.*, 1998, Tinland *et al.*, 1995, Ziemienowicz *et al.*, 2000). Considering our data, where the presence of a very different heterologous C-terminal sequence derived from VirF allowed for quite efficient transient and stable transformation events, even by severely truncated VirD2 proteins, it can be concluded that the C-terminal part of VirD2 is not of any special importance for T-DNA integration. Altogether, it is much more likely that the region C-terminal of amino acid 204 functions as a complex translocation signal, having at least two components: one for interaction with the T4SS and the other, we hypothesize, to enable recruitment of the T-complex to the T4SS.

For VirD2, several interactions responsible for recruitment of VirD2 and the T-strand to the T4SS within *A. tumefaciens* cells have recently been reported: with the VirD2-binding proteins (VBP) 1 to 3 (Guo *et al.*, 2007a and b) and with VirC1 (Atmakuri *et al.*, 2007). For the VBP, no additional function is known, but VirC1 is also responsible for enabling the generation of a high number of T-strands (Atmakuri *et al.*, 2007). Our experiments clearly demonstrated that the amino acid sequence corresponding to residues 204 to 264 of the VirD2 protein was able to strongly enhance the ability of VirD2 mutant proteins regarding T-strand transfer (Table 3). This effect was not further enhanced by a more extensive area of the DUF containing amino acids 204 to 373. Therefore, we hypothesize that the amino acids 204 to 264 provide an important interaction site of VirD2 with one or more *A. tumefaciens* proteins. Although binding of VBP1-3 was roughly mapped to the N-terminal half of VirD2 (Guo *et al.*, 2007a), it probably still includes amino acid residues of the 204-to-264 region. The VirD2 region responsible for the interaction with VirC1 is currently unknown. The presence of two signals in VirD2, one for translocation and one for recruitment to the T4SS, is interesting. We hypothesize that this could provide the bacterium with an extra measure of control, preventing the translocation of VirD2 without a T-strand attached. Of the DUF of VirD2, amino acids 265 to 373 seem to be dispensable in both *A. tumefaciens* and the two plant species used in this study. However, we cannot exclude that this particular sequence represents a host range factor in other plant species.

## Acknowledgments

We thank C. N. Fontaine for technical assistance and A. Vergunst for advice in the early stage of the project.

## Materials & methods

### Construction of VirD2 mutants

Cloning steps were performed according to standard techniques in *Escherichia coli* DH5 $\alpha$ . Both *E. coli* and *A. tumefaciens* were cultured in LC (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 8/liter) with the appropriate antibiotics. Primer and DNA oligomer sequences are listed in Table 4.

### Construction of pSKN

Bluescript plasmid pSK+ was digested with *SacI* and *KpnI*, removing the existing polylinker sequences. Four primers (A to D) were used to construct a novel polylinker: A, *SacI-XhoI* F; B, *SacI-XhoI* R; C, *XhoI-KpnI* F; and D, *XhoI-KpnI* R. *NotI* sites flanking the former *SacI* and *KpnI* sites can be used to isolate *NotI* fragments for ligation into pBFF or pBFFstop (discussed below) with conservation of reading frame in the correct orientation. Next to the *NotI* site and just upstream of polylinker I, an 11-codon sequence encodes the SV40 nuclear localization signal (Fig. 4, sequence boxed) (Dunn *et al.*, 1988).

### Construction of VirD2 truncations and modifications in pSKN

For construction of pSKN-VirD2N, the complete open reading frame (ORF) of VirD2 without the stop codon was obtained by polymerase chain reaction (PCR) on DNA isolated from *Agrobacterium* LBA1115 (Hood *et al.*, 1993) using primers D2NheF and D2XhoR. The purified 1.3-kb DNA fragment was digested with *NheI* and *XhoI* and cloned in similarly digested pSKN, creating pSKN-VirD2. The VirD2 truncation mutants were created using this construct. By digestion with *NdeI* and *XhoI*, a 693-bp C-terminal fragment was removed from the VirD2 coding region of pSKN-VirD2 and the desired mutated VirD2 proteins were reconstituted by supplying different *NdeI/XhoI* fragments, with these sites or appropriate overhangs provided via primer sequences. For the creation of VirD2-204, the *NdeI/XhoI* gap in pSKN-VirD2 was filled in with a double-stranded oligo with *NdeI/XhoI*-compatible overhangs using annealed VirD2-204FW and VirD2-204 RV. VirD2-264 was

made by filling of the *NdeI/XhoI* gap with a *NdeI/XhoI*-digested PCR fragment, made with primers D2FW and D2-264RV. VirD2-373 was made similarly by filling the gap with the *NdeI/XhoI*-digested PCR-product from primers VirD2 FW and VirD2 373 RV.

To create versions of VirD2-204, VirD2-264, and VirD2-373 that were complemented with C-terminal sequences from VirD2, pSKNVirD2 truncations were digested with *XhoI* and *EcoRI*. The same sites were present in all pairs of oligo sequences used. Between the end of the VirD2 truncations and the C-terminal sequences, a sequence encoding the small amino acid linker GGGGS was inserted to secure flexibility of the resulting structures (Wriggers *et al.*, 2005). In this set of constructs, no translated polylinker sequences were present, unlike in pBFF constructs (discussed below). Klenow extension of annealed oligos was used to create these constructs, essentially as described (Uhlman, 1988), followed by *XhoI/EcoRI* digestion of the double-stranded DNA fragments: pSKNVirD2-204/264/373 C-term- $\Delta\omega$ , LINLSTSFV and LINLSTSRV; and pSKNVirD2-204/264/373 NLS- $\omega$ , LINLSOFV and LINLSORV.

### **Construction of *A. tumefaciens* expression vectors pBFF and pBFFstop**

For expression of the different VirD2 proteins in *A. tumefaciens*, the vectors pBFF and pBFFstop were constructed. The wide-host-range, non-mobilizable plasmid pBFF was based on plasmid pRL662 (Vergunst *et al.*, 2000) and constructed as follows: an undesirable *XhoI* site in the plasmid was removed by Klenow fill-in to give pBBR6 $\Delta$ Xho. This vector was digested with *NotI* and the 5' overhangs were filled in with T4 DNA polymerase in the presence of dNTPs. After heat inactivation of the enzyme, vector DNA was precipitated and digested with *XmaI*. A 1,300-bp *XmaI/ScaI* fragment containing the promoter and the complete ORF of the VirF gene sequence of the octopine type *A. tumefaciens* (Ti plasmid pTiB6) was cloned into the vector to give pBBR6 $\Delta$ XhoVirF. Subsequently, most of the VirF coding sequence was removed by digestion with *BspHI*, which cuts in the VirF ATG translational start codon, and *XhoI*, which cuts just upstream of the 37-amino-acid VirF C-terminal T4SS transfer domain (Vergunst *et al.*, 2000). A double-stranded FLAG oligomer consisting of annealed FLAG NotFW and FLAG-NotRV was digested with *NcoI* and *XhoI* and cloned into the *BspHI/XhoI*-digested vector to give the final plasmid pBFF, which thus combines a unique *NotI* cloning site between an N-terminal FLAG-tag and F (Fig. 3). In pBFF, the truncated VirD2 protein was expressed as FLAG-NLSVirD2 truncation F, including 30 amino acids between the VirD2 truncation and F as a result of translation of

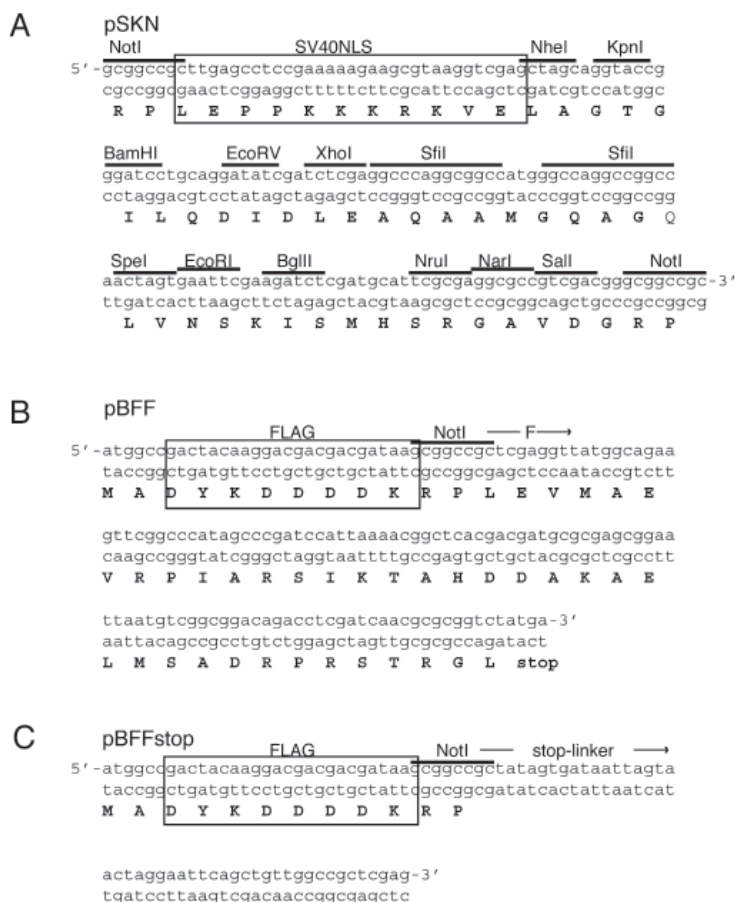
polylinker sequences from pSKN. In pBFFstop, the stop codon was in the polylinker of pBFF, resulting in proteins that end on 31 random amino acids encoded by the polylinker. The presence of this random amino acid sequence did not affect transformation efficiency (data not shown). All FLAG-NLS-VirD2 constructs were expressed under the control of the *virF* promoter, which performed equally well as the *virD* promoter (data not shown). To create pBFFstop, pBFF was cut with *NotI* and a double-stranded *NotI*-compatible DNA fragment harboring several stop codons was introduced at this site, restoring the single *NotI* site that was originally present but prohibiting any in-frame read-through into the VirF C-terminal sequence. The oligos annealed to generate this fragment were FW stop and RV stop.

To create pBRRVirD2, which expresses VirD2 protein under control of the *virF* promoter, without any N- or C-terminal fusions, pBBR6ΔXhoVirF was digested with *BspHI*, treated with Klenow to fill in recessed ends, and subsequently digested with *XhoI*. A PCR product was generated with the primers D2StuIFW and D2XhoIRV. The amplicon was digested with *StuI* and *XhoI* and inserted into the vector. pBFF-VirD2stop, which expresses an N-terminally FLAG and SV40 NLS-tagged VirD2 protein with a wild-type C-terminal sequence, thus without any VirF sequences or amino acids arbitrarily encoded by polylinker sequences, was created by digesting pBFFstopVirD2 and pBRRVirD2 with *DrdI* and *RsrII*. The 4,413-bp fragment of pBRRVirD2 was then ligated to the 1,240-bp *DrdI/RsrII*-fragment of pBFFVirD2, restoring the VirD2 ORF.

pSDM3149 (A. Vergunst, A. Den Dulk-Ras, and P. J. J. Hooykaas, *unpublished*) was based on plasmid pRL662 (Vergunst *et al.*, 2000). Essentially, it is a wide-host-range, non-mobilizable plasmid containing the *virD* promoter and *virD2* gene as amplified from Ti plasmid pTiA6 (Rossi *et al.*, 1993). For root transformation experiments, the plasmids were transferred to *A. tumefaciens* LBA2585 (GV3101, pPM2260, Δ*virD2*, and ΔT-DNA) (Bravo-Angel *et al.*, 1998) by electroporation. Vector pCAMBIA2301 was used as T-stand donor (T-DNA contains GUS::intron and the kanamycin resistance marker). pCAMBIA2301 was transferred to LBA2585 by triparental mating (Ditta *et al.*, 1980).

### **Root transformation assay in *Arabidopsis thaliana* ecotype C-24**

Root transformation experiments were performed as described earlier (Vergunst *et al.*, 2000). In short, seedlings of *Arabidopsis thaliana* ecotype C-24 were grown for 10 days in liquid medium, after which roots were harvested and pre-incubated for 3 days on



**Figure 4:** **A**, pSKN; **B**, pBFF; and **C**, pBFFstop vectors. Amino acids encoded by the top strand are indicated below the double-stranded sequence.

medium containing 2,4-dichlorophenoxyacetic acid at 0.5 ml/liter and kinetin at 0.05 mg/liter, followed by cocultivation with *Agrobacterium tumefaciens* LBA2585 containing the appropriate VirD2 donor vector and pCAMBIA2301 as T-DNA donor for 2 days on solid medium containing 2,4-dichlorophenoxyacetic acid at 0.5 ml/liter, kinetin at 0.05 mg/liter, and 20  $\mu$ M acetosyringone. After this period, roots were either stained for GUS activity or placed on medium containing N6-(isopropenyl)adenine at 5 mg/liter and indole 3-acetic acid at 0.15 mg/liter and, for selection, kanamycin at 50 mg/liter and timentin at 100 mg/liter to kill remaining *A. tumefaciens*.

Kanamycin-resistant calli were counted either after 2 and 3 weeks (WT VirD2) or after 3 and 4 weeks (others) for several independent experiments (more details can be found in the figure captions). The highest numbers of the pairs of calli counts were used

for further calculations regarding the potency of different mutants to lead to transfer and integration of T-strands. In that manner, obvious false differences were excluded because these were predominantly caused by fusion of closely neighboring calli on root segments in positive controls, which should lead to an underestimation of callus formation. Data were normalized against values for WT VirD2 (from *A. tumefaciens* LBA2585 containing pSDM3149 and pCAMBIA2301).

For the determination of transient transformation events, at least 100 root explants per construct per experiment were stained for a few hours or overnight in staining solution containing X-gluc after 3 days of cocultivation with *Agrobacterium* strains.

### **Western blot assay**

*Agrobacterium* cultures were induced with 3  $\mu$ M acetosyringone for at least 4 h before being harvested. A crude protein extract was made by boiling *Agrobacterium* cells in Laemmli buffer with  $\beta$ -mercapto-ethanol for 10 min. Insoluble proteins and debris were spun down and the supernatant was used for Western blotting. Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membrane, and treated with FLAG antibody conjugated to alkaline phosphatase (cat no. A9469; Sigma- Aldrich). Detection of alkaline phosphatase activity was performed using nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate solution (Sigma-Aldrich) according to instructions from the manufacturer.

**Table 4.** Primer and DNA oligomer sequences**Primer Sequence<sup>a</sup>**

A ( <i>SacI</i> - <i>XhoI</i> F)	GCGGCCGCTTGAGCCTCCGAAAAAGAGCGTAAGGTCGAGCTAGCGGTACCGGGA- TCCTGCAGGATATCGATCTCGAGGCC
B ( <i>SacI</i> - <i>XhoI</i> R)	GGGCCTCGAGATCGATATCCTGCAGGATCCCGGTACCTGCTAGCTCGACCTTACG- CTTCTTTTTTCGGAGGCTCAAGCGGCCGCCAGCT
C ( <i>XhoI</i> - <i>KpnI</i> F)	TCGAGGCCCAGGCGCCATGGGCCAGGCCGCCAACTAGTGAATTCGAAGATCT CGATGCATTCGCGAGGCGCCGTCGACGGGCGGCCGCGTAC
D ( <i>XhoI</i> - <i>KpnI</i> R)	GCGGCCCGCCGTCGACGGCGCCTCGCGAATGCATCGAGATCTTCGAATTCACTA GTTGGCCGGCCTGGCCCATGGCCGCTGGGCC
D2NheF	GCGAGCTAGCGATGCCCCGATCGCGCTCAAG
D2XhoR	GCGACTCGAGGGTCCCCCGCGCCCATC
D2FW	CCTGGATGCGACTTCGCGAGC
D2-264RV	CATACTCGAGGGACTCCTGAAAACGGGCG
VirD2-373RV	CATACTCGAGGCGAGTGTCTTGCTCACCGAC
LiNLSFSFW	GATCCTCGAGGGAGGTGGTGAAGTAAGCGTCCGCGTGACCGTCACGATGGGAA TTGGGTG
LINLSRSRV	CTAGGAATTCCTAGCGACGATTACCTCTTGACAGTTTGCGTCCACCCAATTCTCCA TCGTGA
LINLSOFW	GGATCCTCGAGGGAGGTGGTGAAGTAAGCGTCCGCGTGACCGTCACGATGGA GAATTGGGTGGAC
LINLSORV	CCTAGGAATTCCTAGGTCCCCCGCGCCCATCTCTTGACAGTTTGCGTCCACCC AATTCTCCATCG
VirD2NotI	GGATCGCGGCCGCTTGAGCTAGCGATGCCC
VirD2RV	CCATGGCGGCCGCCGTCGACGGCG
FLAG-NotFW	GACGCTCTGTTTCTCTCACCACAGCCATGGCCGACTACAAGGACGACGACGATAA GCGGCCGCTCGAGACTAGTGAGCT
FLAG-NotRV	ACTAGTCTCGAGCGGCCGCTTATCGTCGTCGTCCTTGTAGTCGGCCATGGCTGTG GTGAGAGAAACAGAGCGTCAGCT
FW <sub>stop</sub>	GGCCGCTATAGTGATAATTAGTAACTAGGAATTCAGCTGTT
RV <sub>stop</sub>	GGCCAACAGCTGTATTCCTAGTTACTAATTATCACTATAGC
D2StuI FW	GAAGGCCTGATCGCGCTCAAGTAATCATTCGC
D2XhoI RV	GACTCGAGCTAGGTCCCCCGCGCCCAT

<sup>a</sup>Initial VirD2 truncations were created in the high copy vector pSKN (a bluescript derivative) and subcloned into destination vectors pBFF and pBFF<sub>stop</sub> as *NotI* fragments.

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