Virulence protein VirD5 of *Agrobacterium tumefaciens* binds to kinetochores in host cells via an interaction with Spt4

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The bacterium *Agrobacterium tumefaciens* causes crown gall tumor formation in plants. During infection the bacteria translocate an oncogenic piece of DNA (transferred DNA, T-DNA) into plant cells at the infection site. A number of virulence proteins are cotransported into host cells concomitantly with the T-DNA to effectuate transformation. Using yeast as a model host, we find that one of these proteins, VirD5, localizes to the centromeres/kinetochores in the nucleus of the host cells by its interaction with the conserved protein Spt4. VirD5 promotes chromosomal instability as seen by the high-frequency loss of a minichromosome in yeast. By using both yeast and plant cells with a chromosome that was specifically marked by a *lacO* repeat, chromosome segregation errors and the appearance of aneuploid cells due to the presence of VirD5 could be visualized in vivo. Thus, VirD5 is a prokaryotic virulence protein that interferes with mitosis.

Agrobacterium | kinetochore | Spt4 | mitosis | virulence

The plant pathogen *Agrobacterium tumefaciens* naturally causes crown gall disease in dicotyledonous plants, which is characterized by the formation of plant tumors at infection sites (1). During infection, a single-stranded copy of a segment of the bacterial tumor-inducing (Ti) plasmid, called the "T-strand," is transferred and integrated into the host genome (1–3). Expression of the genes present in this transferred DNA (T-DNA) in transformed plant cells results in uncontrolled cell division and development of a crown gall tumor (1). Besides the T-region, the Ti plasmid embraces an area called the "virulence region," which contains a set of genes that are essential for virulence of the bacterium and which mediate T-DNA processing and its delivery into host cells (4, 5). Concurrently with the T-strand, some of these virulence (Vir) proteins, including VirD2, VirE2, VirE3, VirF, and VirD5, are translocated into plant cells via the VirB type IV secretion system of the bacterium (6, 7). The translocated ssDNA-binding protein VirE2 is thought to coat the T-strand in the host cell cytoplasm, forming a T-complex that is protected against nucleases (8, 9). VirE2 also interacts with a host protein called "VIP1" (10), a transcription factor which moves to the nucleus as part of the defense response (11), and this may promote transfer of the T-complex to the nucleus (10, 11). More recently, however, the importance of VIP1 in transformation was questioned, as a *vip1* mutant was transformed equally well as the wild type (12). The VirF protein is a host range factor (13) which contains an F-box and thus may be incorporated into a Skp1-Cdc53-F-box (SCF) ubiquitin-ligase (E3) complex in the host cells (14). The VirF SCF complex is thought to promote the proteolytic degradation of VirE2, VIP1, and some other host proteins in the nucleus (15, 16). This may lead to uncoating of VIP1 and VirE2 from the T-strand and facilitate T-DNA integration (16). Proteolysis of VIP1 may at the same time dampen the defense response by VIP1 (17). An endogenous F-box protein called "VBF" present in some host plants such as *Arabidopsis thaliana* may take over from VirF, and in such plants VirF is not needed for transformation (18). The transported VirE3 protein is imported into the host cell nucleus, where it interacts with pBrp, a TFIIB-like transcription factor, and stimulates transcription of host genes including *VBF* (19, 20). This explained why the simultaneous deletion of *virF* and *virE3* led to much stronger attenuation of virulence than seen in the single mutant (19). The translocated VirD5 protein consists of 833 amino acids and contains two putative nuclear localization signals (NLSs), and putative helix–turn–helix and helix–loop–helix domains (21). The function of VirD5 is still unknown, but in its absence tumor formation is attenuated (22, 23). It was reported that VirD5 stabilizes VirF in host cells via mutual interaction (22) but also that VirD5 is a nuclear competitor of VBF for binding to VIP1 to stabilize VIP1 and VirE2 (23).

We found that VirD5 was toxic for both plant and yeast cells, which indicated that VirD5 may target a conserved essential process. By using the yeast *Saccharomyces cerevisiae* as a model organism, we established that VirD5 binds to the chromosomal centromeres/kinetochores in the nucleus by interacting with the conserved kinetochore-associated protein Spt4. This leads to growth inhibition and chromosome missegregation in both yeast and plants and the appearance of aneuploid cells.

Results

VirD5 Inhibits Growth of Plant and Yeast Cells. To obtain more insight into the function of VirD5, we expressed the protein in *A. thaliana*. To this end, a binary vector containing the *virD5* gene driven by a tamoxifen-inducible promoter was transformed into *A. thaliana* via flora dip. Fifteen independent transformed plants were propagated on kanamycin-selection medium. To test whether VirD5 can influence plant growth and development, T2 seeds from each of these lines were germinated on kanamycin medium to which tamoxifen had been added at 1 μM or 10 μM to induce the expression of VirD5 or in the absence of tamoxifen. In the presence of tamoxifen seedlings died within 2 wk, but without

Significance

The soil bacterium *Agrobacterium tumefaciens* transforms plant cells into tumor cells by transferring a segment of oncogenic DNA (T-DNA). During transformation a set of virulence proteins is translocated into the plant cells to promote transformation. We show here that one of these proteins, VirD5, has the capacity to interfere with the conserved process of mitosis. By binding to the conserved host protein Spt4, VirD5 accumulates in the nucleus at the centromeres/kinetochores. There it can cause chromosome missegregation generating aneuploid cells in both yeast and plant cells.

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tamoxifen the transgenic seedlings showed normal growth (Fig. 1*A*). This result suggested that VirD5 might target an essential cellular process. The yeast *S. cerevisiae* is an excellent model to analyze the function of bacterial effector proteins that in nature exert their function in multicellular eukaryotes (24, 25). To determine if we could take advantage of the yeast system, the *virD5* gene was cloned into a yeast multicopy plasmid behind the galactose-inducible *GAL1* promoter and was transformed into strain BY4743. Transformed cells were grown on MY medium containing 2% glucose for 3 d, and thereafter colonies were taken from the plates, suspended, and serially diluted and spotted onto an MY plate containing either 2% glucose (MYglu) or 2% galactose (MYgal), which was incubated for another 3 d. Presence of the VirD5 expression construct (from several different *Agrobacterium* strains) prevented growth in yeast (Fig. 1*B*), whereas a comparable construct with a frameshift did not inhibit growth (Fig. 1*B*). Toxicity depended on the expression level, which could be modulated by the choice of sugar (inducer) present in the growth medium (Fig. S1). The conservation of the toxicity allowed us to use yeast genetics to study the underlying molecular mechanisms. We screened a genomic deletion library for deletion mutations that suppress the toxic effects of VirD5, as such suppressors may reveal the identity of the target of VirD5. A high-copy plasmid containing the *virD5* gene under the control of the *GAL1* promoter (pMVHis-VirD5) was transformed into all of the ≈5,000 viable yeast deletion strains. After growing on MYglu plates for 3 d, colonies were streaked onto MYgal plates and incubated for an additional 3 d. Most of these transformants could not survive on MYgal plates due to the toxicity of VirD5, but 33 deletion mutants showed some growth. Upon reanalysis of these individual deletion mutants, three still showed (partial) suppression of the toxicity of VirD5. In two of these mutants, genes (*GAL3* and *GAL4*, respectively) were deleted, compromising the transcriptional activation of the *GAL* genes, and thus in these mutants the expression of VirD5 was prevented, explaining their survival. This also shows the effectiveness of the selection strategy. The other deletion strain that showed suppression of the toxicity of VirD5 had a deletion in the *SPT4* gene (Fig. 1 *C* and *D* and Fig. S2), the product of which may be a potential target of VirD5, may stabilize or enhance the level of VirD5 in the cell, may influence the location of VirD5 in the cell, or otherwise may be necessary for the toxicity of VirD5. To confirm that the deletion in this strain was responsible for the suppression of the toxicity of VirD5, the plasmid isolated from the mutant was retransformed into wild-type yeast cells. This analysis showed that the construct encoding VirD5 obtained from the *spt4Δ* mutant had kept its toxicity (Fig. S2*B*). This indicated that the presence of *SPT4* was essential for the toxicity of VirD5. Whereas colony formation on medium containing galactose was prevented in yeast strain BY4743 expressing VirD5, the isogenic strain with the *SPT4* deletion formed similar numbers of colonies irrespective of VirD5 expression (Fig. S2*C*).

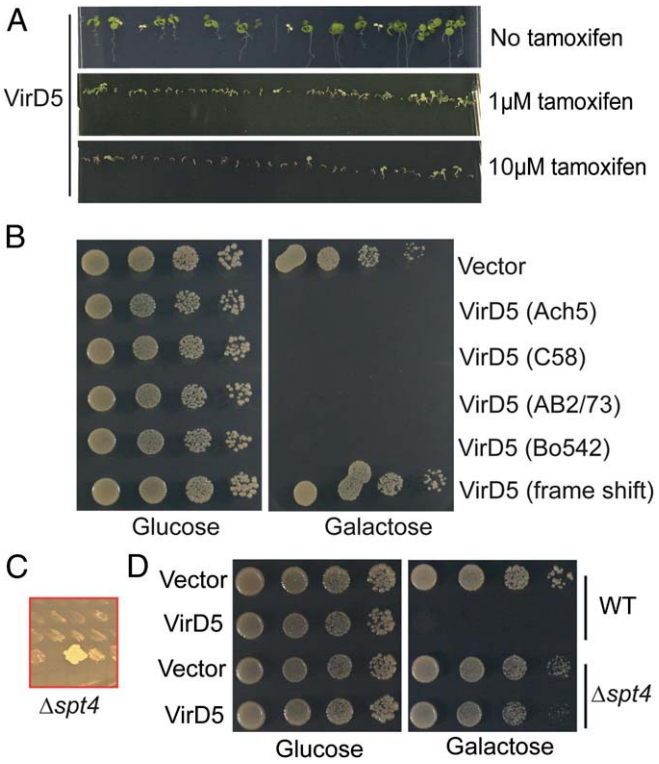

Fig. 1. VirD5 inhibits the growth of plants and yeast. (*A*) Transgenic *A. thaliana* containing *virD5* driven by the tamoxifen-inducible promoter were grown on MS medium containing different concentrations of tamoxifen or without tamoxifen (DMSO). Kanamycin was present to select for seedlings containing T-DNA. (*B*) Yeast cells (BY4743) transformed with a high-copy plasmid (pMVHis-VirD5) encoding VirD5 from different *Agrobacterium* strains under the control of the *GAL1* promoter. Transformants were fivefold serially diluted and spotted onto MY selection medium containing either 2% glucose or 2% galactose and were incubated for 3 d at 30 °C. (*C*) Identification of *spt4*Δ as a suppressor of VirD5 toxicity. A representative portion of the whole genome-wide deletion collection (~5,000 strains) was transformed with plasmid (pMVHis-VirD5). Transformants were first selected on glucose medium and then were streaked onto galactose plates. (*D*) Wild type or the *spt4*Δ-deletion mutant transformed with a high-copy empty plasmid pMVHis or pMVHis-VirD5. Transformants were fivefold serially diluted, spotted onto MY selection medium containing either 2% glucose or 2% galactose, and incubated for 4 d at 30 °C.

Subcellular Localization of VirD5. We fused VirD5 N-terminally with GFP (GFP-VirD5) to study its localization and expressed it from the *MET25* promoter on a single-copy plasmid in strain BY4743:HTA2-CFP, in which the nucleus was marked by labeling of histone H2A with cyan fluorescence. Cells with the plasmid could grow when the expression of GFP-VirD5 was blocked by the presence of methionine and also for a short period after removal of methionine. This allowed us to visualize green fluorescence under a confocal microscope in these cells 1 h after removal of methionine. While GFP fluorescence was present throughout the cell in control cells expressing unfused GFP, GFP-VirD5 was seen clustered as bright dots in the nucleus (Fig. 2*A*), indicating that VirD5 is localized at specific foci in the nucleus. The genome-wide deletion mutants screening indicated that deletion of *SPT4* reduced the toxicity of VirD5. Crotti and Basrai (26) showed that a Spt4-GFP fusion protein is localized to three to seven foci in the yeast nucleus, a pattern resembling that seen with the GFP-VirD5 fusion. Some of the Spt4-GFP foci have been shown to overlap with kinetochore-containing Ndc10-HA foci, indicating that a subset of Spt4-GFP foci localize at the kinetochores, where Spt4 contributes to the formation of the centromeric chromatin structure and to chromosome transmission fidelity (26). This suggested that VirD5 might similarly be targeted to centromeres/kinetochores and that its toxicity may be due to impaired chromosome segregation.

To determine whether VirD5, like Spt4, is localized at the centromeres/kinetochores, the centromere/kinetochore-associated proteins Ndc10 and Spt4 were fused N-terminally with CFP in a construct driven by the *MET25* promoter and subsequently cotransformed with a construct expressing GFP-VirD5 into yeast. Cells were observed under a confocal microscope 1 h after the removal of methionine. GFP-VirD5 foci overlapped fully with both CFP-Spt4 foci and CFP-Ndc10 foci (Fig. 2*B*), suggesting that VirD5, like Spt4, is present at the centromeres/kinetochores that are marked by Ndc10. To test whether VirD5 interacts with Spt4, we performed bimolecular fluorescence complementation (BiFC) experiments (27). VirD5 was fused with the C-terminal part of YFP (VC173) and transformed into BY4743 cells together with Spt4

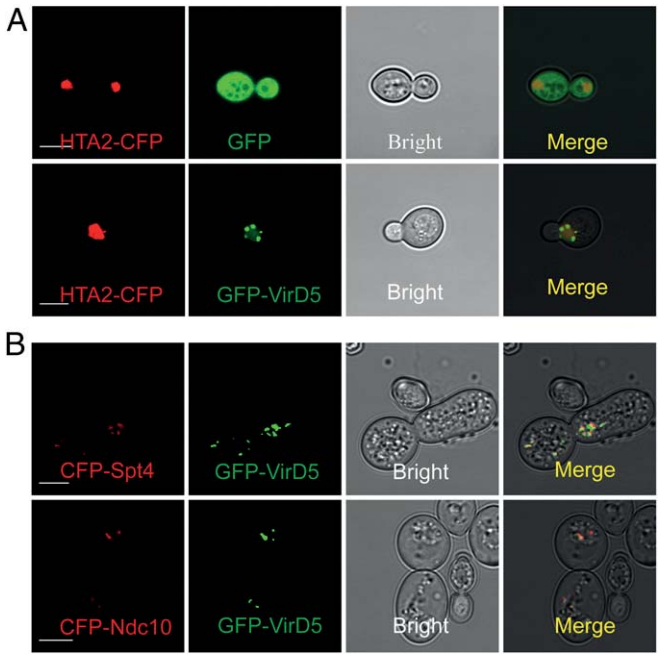

Fig. 2. VirD5 colocalizes with the centromere/kinetochore-associated proteins Spt4 and Ndc10. (*A*) Yeast cells (BY4743:HTA2-CFP) transformed with plasmid encoding empty GFP or GFP-VirD5. (*B*) Yeast cells (BY4743) transformed with plasmid encoding GFP-VirD5 together with plasmid encoding CFP-Spt4 or CFP-Ndc10. (Scale bars, 5 μm.)

fused with the N-terminal part of YFP (VN173). As can be seen in Fig. 3*A*, VirD5 displayed a very strong BiFC signal with Spt4 in the nucleus, whereas the fusions of VirD5 or Spt4 introduced together with the unfused complementary part did not give a BiFC signal. To confirm this interaction, an in vitro pull-down assay was done with GST-Spt4 bound to the glutathione HiCap matrix using unfused GST as a control. The beads were incubated separately with His-tagged VirD5, and after three washings the protein mixtures were separated on a 10% SDS/PAGE gel. As shown in Fig. 3*B*, VirD5 copurified with GST-tagged Spt4 but not with empty GST, suggesting that Spt4 binds directly to VirD5.

As deletion of *SPT4* suppressed the toxicity of VirD5, we investigated whether this deletion led to an altered localization of VirD5. As shown in Fig. 3*C* GFP fluorescence was present throughout the nucleus in the *spt4*Δ-deletion strain, and we could not find foci in over 90% of transformed cells. Only a few foci were present in the remaining 10% of transformed cells. Introduction of a wild-type *SPT4* gene into the Δ*spt4*-deletion strain restored the punctate foci of VirD5 in all the cells (Fig. 3*C*).

The N-Terminal Part of VirD5 Is Responsible for Targeting to the Centromeres/Kinetochores. Protein sequence alignment showed that the N-terminal part of VirD5 contains six degenerate repeats, each of which consists of ≈40 amino acids (Fig. S3). To determine whether these N-terminal repeats are responsible for targeting to the centromeres/kinetochores in yeast cells, the 505 N-terminal amino acids of VirD5 (VirD5NT) embracing these six repeats and, as a control, the 313 C-terminal amino acids of VirD5 (VirD5CT) were fused in frame with GFP and expressed under the control of the *MET25* promoter in wild-type BY4743 cells. After cells were shifted to methionine-free medium for 1 h, dots were seen in the nuclei of cells expressing GFP-VirD5NT but not in those expressing GFP-VirD5CT, where the GFP signal was distributed throughout the cell (Fig. S4*A*). This indicates that the N terminus of VirD5 mediates the accumulation at the centromeres/kinetochores. We also made a construct embracing a smaller N-terminal part, VirD5 (1–202). In contrast to VirD5 (1–505), this construct did not accumulate at the centromeres/kinetochores (Fig. S4*A*). Subsequently, we verified the growth-inhibitory properties of constructs encoding VirD5 (1–202) and VirD5 (1–505) in comparison with full VirD5. As can be seen in Fig. S4*B*, the expression of VirD5 (1–505) in BY4743 yeast cells led to growth inhibition but did not prevent growth altogether like the full protein, suggesting that the C-terminal part also contributes to toxicity. The expression of VirD5 (1–202) did not interfere with growth. To determine whether the toxicity of VirD5NT could be relieved by the deletion of *SPT4*, we transformed either a high-copy empty vector or a vector encoding VirD5NT in either wild-type yeast or the Δ*spt4*-deletion mutant. In these experiments we used VirD5NT fused with three nuclear localization signals (VirD5NT-3×NLS), which was somewhat more toxic than VirD5NT (Fig. S4), probably because more VirD5 enters into the nucleus. In the absence of

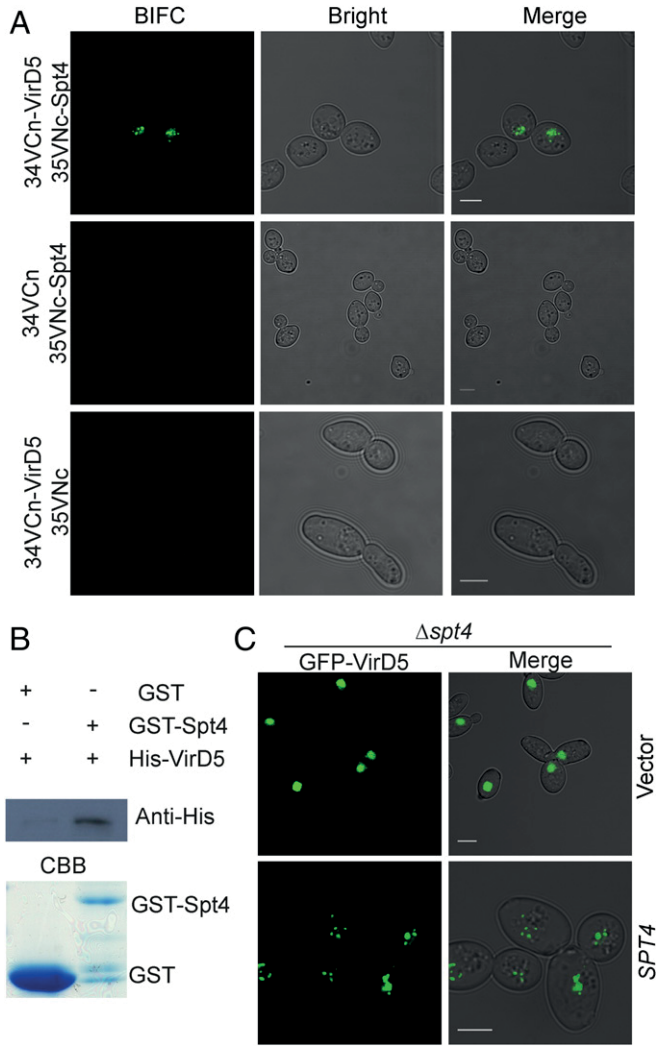

Fig. 3. VirD5 physically interacts with Spt4. (*A*) Yeast cells (BY4743) transformed with BiFC vectors. 34VCn, the C terminus of YFP (VC173) fused with the N terminus of the proteins to be tested; 35VNc, the N terminus of YFP (VN173) fused with the C terminus of the proteins to be tested. (*B*) 6×His-tagged VirD5 was incubated with either GST or GST-Spt4 in vitro; after washing, the presence of 6×His-VirD5 bound to the glutathione HiCap matrix was detected by anti-His antibody (His-probe antibody; Santa Cruz). (*C*) The *spt4* deletion mutant was cotransformed with plasmid encoding GFP-VirD5 and a single-copy empty plasmid pRS315 or plasmid (pRS315-Spt4) encoding wild-type Spt4 with its own promoter and terminator. (Scale bars, 5 μm.)

SPT4, VirD5NT-3×NLS was no longer toxic to yeast cells (Fig. S4*C*), suggesting that Spt4 is required for VirD5NT-3×NLS to exert its toxicity. To test whether VirD5NT interacts with Spt4, we carried out a BiFC assay. As can be seen in Fig. S3*B*, VirD5NT showed a clear BiFC signal with Spt4. These data indicate that Spt4 binds to the N-terminal part of VirD5 and thus localizes it at the centromeres/kinetochores, allowing VirD5 to exert its toxic effect at the centromeres/kinetochores.

VirD5 Causes Chromosome Loss and Missegregation. Centromeres/kinetochores are key platforms for accurate chromosome segregation during mitosis. In view of its location at the centromeres/kinetochores, we wondered whether the presence of VirD5 affects chromosome segregation and leads to chromosome loss. To study this, we used the yeast strain RLY4029 (28), which contains a minichromosome, CF, harboring the *URA3* gene and the *SUP11* gene suppressing red pigment accumulation as a consequence of the chromosomal *ade2-101* mutation. Cells carrying CF produce white colonies, whereas cells lacking CF form red colonies. RLY4029 cells with and without a construct encoding VirD5 under the control of the *GAL1* promoter inserted at the *LEU2* locus were grown first in minimal medium containing 2% glucose but lacking uracil, followed by a shift to yeast extract/peptone (YP) rich medium (with uracil) containing 2% raffinose and 2% galactose for 24 h. The induced cells were serially diluted and plated on YP rich medium containing 2% glucose to repress expression of VirD5. As can be seen in Fig. 4*A*, a >10-fold higher rate of minichromosome loss was observed in cells expressing VirD5 compared with that in control cells. These data indicate that VirD5 causes chromosome instability.

To find out whether this is due to chromosome missegregation, we integrated *virD5* driven by the *GAL1* promoter into the genome of the haploid yeast strain Y716 (29). This strain expresses a GFP-LacI fusion protein that binds to a 256-tandem repeat *lacO* operator array integrated into chromosome I of this yeast strain, thus marking chromosome I with a bright green fluorescent dot inside the nucleus. After culture for 6 h in the presence of galactose, cells lacking VirD5 divided normally and showed a single fluorescent dot in accordance with an equal distribution of chromosome I over the mother and daughter cell. However, ≈97% of the cells expressing VirD5 displayed an erroneous distribution of chromosome I over the mother and daughter cells (Fig. 4*B*). We found three classes of missegregation: The major class represented mother cells with two chromosomes I and daughter cells lacking chromosome I; the second class comprised mother cells with chromosome I (one dot) and daughters lacking chromosome I; and the third class comprised pairs of cells with a total of more than two bright GFP dots (Fig. 4*B*).

Chromosome Missegregation in Plants. As Spt4 is a conserved protein, we tested whether VirD5 also interacted with the two *A. thaliana* Spt4 orthologs in BiFC experiments in yeast cells. As VirD5 indeed also interacted with one of the plant Spt4 orthologs (Fig. S5), we similarly analyzed whether VirD5 could also affect chromosome segregation in plant cells. To this end, we used an *A. thaliana* plant line containing a 256-tandem repeat *lacO* operator array integrated into chromosome 5 that could be visualized under the microscope as a bright green fluorescent dot by expression of GFP-LacI. The cells also expressed a H2B-DsRed fusion protein to mark the nucleus with red fluorescence (30). This chromosome-marked homozygous plant line was crossed with a

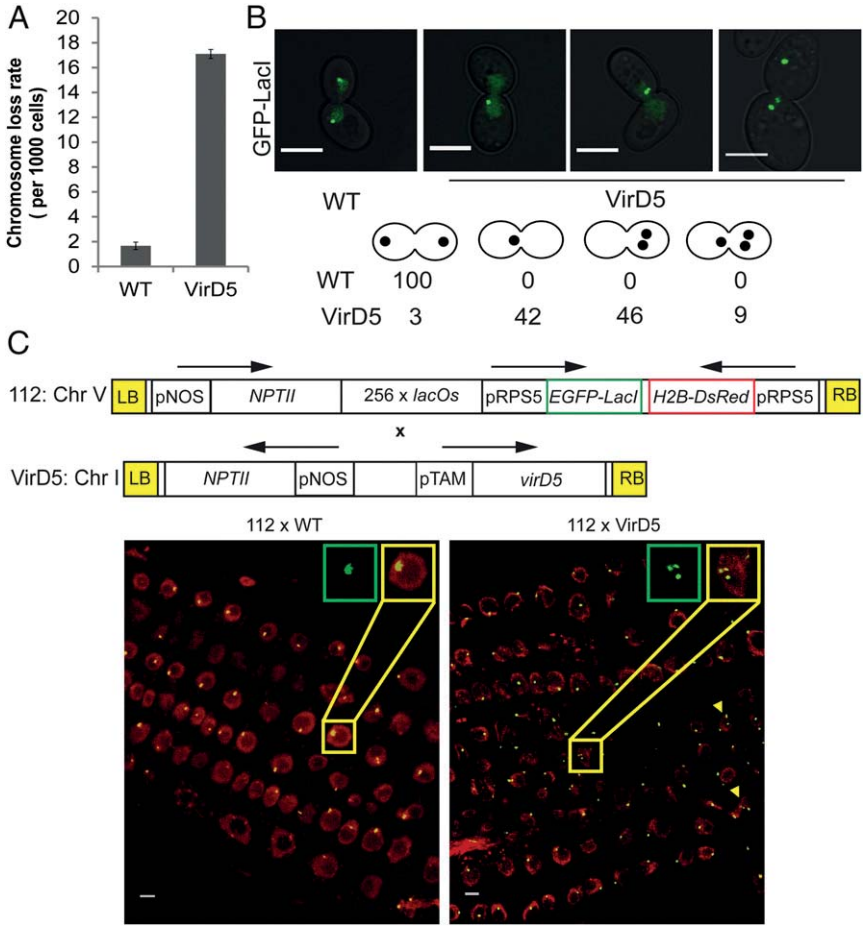

Fig. 4. VirD5 disturbs chromosome segregation. (*A*) The yeast strain RLY4029 contains an artificial minichromosome harboring a gene (*SUP11*) suppressing red pigment accumulation. Loss of this minichromosome was inferred from the frequency of red colonies. Error bars represent the mean ± SD from three independent experiments. (*B*, *Upper*) The yeast strain Y716 contains a 256-repeat *lacO* array in chromosome I and expresses GFP-LacI, allowing visualization of chromosome I by confocal microscopy. After integration of a construct encoding VirD5 under control of the *GAL1* promoter into the genome, cells were cultured in YP medium containing 2% glucose and then were shifted to YP medium containing 2% raffinose and 2% galactose. One hundred mitotic cells with or without VirD5 were observed. (*Lower*) Examples and the numbers of properly and missegregated chromosomes in mother/daughter cells are shown. (*C*) Plant line 112 is a homozygous plant line in which chromosome 5 contains a construct embracing a 256-repeat *lacO* array as well as genes encoding EGFP-LacI and H2B-DsRed under the control of *RPS5A* promoter (pRPS5). "WT" represents the isogenic wild-type plant line. VirD5 represents a homozygous transgenic plant line with a construct encoding VirD5 driven by the tamoxifen-inducible promoter (pTAM). After crossing, the GFP-marked chromosome 5 was visualized by confocal microscopy. Fifty F1 plants from each crossing line were analyzed. (Scale bars, 5 μm.) Transformed plants were initially selected by an NPTII gene driven by the nopaline synthase (pNOS) promoter. The inserted T-DNA is defined by the left (LB) and right (RB) border repeats.

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homozygous transgenic *A. thaliana* line containing the *virD5* gene driven by the tamoxifen-inducible promoter. F1 seeds of this cross were germinated on Murashiga and Skoog (MS) medium without tamoxifen. Subsequently, 4 d after germination, seedlings were moved to liquid MS medium containing 10 μM tamoxifen and were incubated for an additional 24 h. During this period the seedlings still survived. Root cells from the meristematic zone were chosen for analysis because of their lower background fluorescence, and we examined roots from 50 individual plants from each cross. As can be seen in Fig. 4*C*, root cells from F1 heterozygous plants from a cross of the *lacO*/GFP-LacI line with the wild-type plants showed mainly nuclei with a single bright GFP dot, and, in a few mitotic cells before cytokinesis, with two dots. In contrast, in root cells from F1 plants from the cross with the VirD5-expressing line, in addition to many cells with a single bright dot, cells displaying more than two bright dots were visible, illustrating chromosome missegregation and generation of aneuploid cells in plants due to the presence of VirD5.

Discussion

It is known that translocated virulence proteins assist *Agrobacterium* in the process of plant transformation. The functions of these translocated effector proteins are still not fully understood. Here we have focused on the VirD5 protein, a relatively large protein of 833 amino acids with several recognizable domains and motifs (21). Previously, it was reported that transformation is attenuated in the absence of VirD5 and that VirD5 stabilizes another translocated effector protein, VirF (22, 23). When we started our study of VirD5, we found that prolonged VirD5 expression from a strong promoter was toxic both in plant and in yeast cells and led to cell death. When expression was restricted in time, cells could recover, and in this way we could study the molecular mechanism of action of VirD5 in the cell. During transformation in vivo, the dose of VirD5 translocated into cells is presumably much lower than after expression from a gene construct in transgenic cells, and due to natural decay its presence is also limited in time. Therefore, it is likely that the transient presence of limited amounts of VirD5 during the initiation phase of tumorigenesis will not be lethal but may slow down mitosis. This may be beneficial for transformation by increasing the time window for T-DNA integration.

Our results showed that GFP-VirD5 expression in yeast cells displayed specific punctate foci in the nucleus (Fig. 2*A*). A similar pattern was seen previously in cells expressing a Spt4-GFP fusion protein (26). As deletion of *SPT4* suppressed the toxicity of VirD5, this motivated our focus on the protein Spt4, a transcription elongation factor, which forms a heterodimeric complex with Spt5 to regulate mRNA transcription via direct interaction with RNA polymerase II (31). The Spt4 protein also plays a role in chromosome segregation and is a functional and structural component of centromeric heterochromatin (26, 32). A gene for this highly conserved protein is present in the human genome, as well as in that of animals, plants, and other eukaryotes (33–35). We found that the deletion of *SPT4* in yeast reduced the toxicity of VirD5 (Fig. 1 *C* and *D* and Fig. S2), and the VirD5 protein was no longer present at the centromeres/kinetochores in most of the cells in this mutant background (Fig. 3*C*). Further data demonstrated that VirD5 colocalized and physically interacted with Spt4 (Figs. 2*B* and 3 *A* and *B*). The centromere contains a specialized nucleosome that mediates chromosome attachment via the kinetochore to the spindle microtubule. In budding yeast, transcription at the centromere induced by the transcription factor Cbf1, an inner kinetochore protein that binds directly to the centromeric DNA, facilitates the centromere function (36). However, strong transcription over the centromere in budding yeast by locating an artificial strong promoter (*GAL1*) adjacent to the centromere inactivated its function, thereby inducing chromosome missegregation and aneuploidy (37). As the VirD5 protein not only is present at centromeres/kinetochores by interaction with Spt4 (Fig. 3 *A* and *B*) but also has transcriptional activation activity (23), it is possible that the toxic effects of VirD5 are (partially) due to erroneous transcription at the centromeres. We could still see a few nuclear foci in 10% of the cells deleted for *SPT4*, suggesting that there may be another protein at the kinetochore that binds VirD5, although with lower affinity than Spt4. Loss of these 10% of the cells by cell death would not be noticed when examining growth in liquid or on plates.

Continued presence of VirD5 leads to chromosome missegregation and aneuploidy, which explains its toxicity and lethality. Aneuploidy is a hallmark of tumor cells in human and mammals (38) and is also characteristically seen in plant crown gall tumor cells (39). However, it remains to be seen whether the levels of VirD5 transferred during infection are sufficient to induce aneuploidy. When used for transgenesis, putative aneuploid cells may not be able to regenerate into plants and therefore are possibly not seen during *Agrobacterium*-mediated transformation of plants. Such cells might, however, contribute to the formation of a crown gall tumor. It has to be seen whether and how the properties of VirD5 described here are related to the previously described activities related to stabilization of VIP1 and/or VirF (22, 23). However, VirD5 is a large protein, and, like other virulence proteins such as VirE2, it may have multiple functions in the transformation process.

Materials and Methods

Primers, Strains, and Plasmids. Primers, strains, and plasmids used in this study are listed in Tables S1, S2, and S3, respectively.

Plant Material. Binary vector pGPINTAM-VirD5 containing *virD5* under the control of a tamoxifen-inducible promoter was transferred into *A. tumefaciens* strain AGL1. *A. thaliana* ecotype Columbia-0 (Col-0) was used for floral dip (40). A few weeks after dipping, mature seeds were harvested and sowed on MS medium [2.3 g/L MS medium including vitamins (Duchefa), 0.5 g/L Mes, 7 g/L agar, pH, 5.8] containing 50 mg/L kanamycin. Kanamycin-resistant T1 transgenic seedlings were checked for the insert by PCR and transferred to soil. T2 seeds from 15 independent T1 transgenic plants were germinated on MS medium containing kanamycin and either DMSO or different concentrations of tamoxifen (dissolved in DMSO) to induce the expression of VirD5. Plant line 112, in which chromosome 5 contains a construct embracing 256 repeats of the *lac* operator as well as genes encoding EGFP-LacI and H2B-DsRed under the control of the *RPS5A* promoter, was obtained from Antonius Matzke, Gregor Mendel Institute, Vienna.

Subcellular Localization of VirD5 and Its Truncations. Plasmids pUG34GFP, pUG34GFP-VirD5, pUG34GFP-VirD5 (1–202), pUG34GFP-VirD5NT (1–505), and pUG34GFP-VirD5CT (521–833) were transformed into yeast BY4743. Transformants were grown at 30 °C on solid MY medium (41) containing histidine and leucine and 30 mg/L methionine to suppress the expression of VirD5 and its truncations. Three days after transformation, colonies were transferred to MY liquid medium containing 30 mg/L methionine. Overnight cultures were diluted and grown at 30 °C in fresh MY liquid medium lacking methionine to induce the expression of VirD5 and its truncations for 1 h. GFP (excitation, 488 nm; emission, 520 nm) and CFP (excitation, 458 nm; emission 475–515 nm) signals were visualized using a 63× oil objective on a Zeiss Imager confocal microscope. Images were processed with ImageJ (NIH) and Photoshop (Adobe).

BiFC Assay. The pUG34VCn-VirD5 plasmid or the empty vector pUG34VCn were transformed with either the empty pUG35VNc vector or a derivative with a cloned putative interaction partner into yeast cells. Transformants were grown at 30 °C on solid MY medium containing 30 mg/L methionine to inhibit the expression of VirD5. After 3 d colonies were transferred to MY liquid medium containing methionine. Cells from overnight cultures were washed twice with water and resuspended in MY medium lacking methionine to induce the expression of VirD5 and other cloned genes. After induction for 1 h, the BiFC signal was analyzed by confocal microscopy (excitation, 514 nm; emission, 522–532 nm) using a 63× oil objective on a Zeiss Imager confocal microscope. Images were processed with ImageJ (NIH) and Photoshop (Adobe).

Chromosome Loss Assay. Strain RLY4029 contains a minichromosome consisting of a fragment of yeast chromosome III with the *SUP11* and *URA3* marker genes (28). The genetic background of this haploid strain carries an

ade2-101 mutation and therefore forms red colonies. The red pigment accumulation is suppressed by the expression of *SUP11* present in the minichromosome, resulting in white colonies. The frequency of loss of this minichromosome can therefore be calculated by counting the numbers of red colonies among the total number of colonies. Plasmid pRS305 with the *virD5* gene under control of the *GAL1* promoter was integrated at the chromosomal *LEU2* locus of strain RLY4029, generating strain RLY4029:VirD5 that can grow on MY with 2% glucose without leucine and uracil. Parental and VirD5-containing yeast cells were cultured overnight in MY glucose selection medium lacking uracil at 30 °C. Cells were diluted and recultured in MY glucose liquid medium without uracil for an additional 6 h. After that, cells were diluted 50-fold and switched to rich medium YP (10 g/L yeast extract, 20 g/L Bacto Pepton) containing 2% raffinose and 2% galactose for 24 h at 30 °C to induce VirD5. Cells cultured overnight were diluted to an appropriate density and plated onto rich medium YP containing 2% glucose for 3 d at 30 °C. Plates were kept at 4 °C for accumulation of red pigment. Total white and red colony numbers were counted.

Chromosome Segregation Assay. Strain Y716 contains a construct encoding GFP-LacI and a 256-repeat *lacO* array integrated in chromosome I (29). Binding of GFP-LacI to the *lacO* repeats allows the visualization of chromosome I as a GFP dot by microscopy. Y716 and derivatives with a chromosomally integrated plasmid pRS306 construct containing the *virD5* gene driven by the *GAL1* promoter were cultured in YP rich medium containing 2% glucose. Cells cultured overnight were diluted to an OD_{620} of 0.1 and recultured in YP rich medium containing 2% raffinose and 2% galactose for an additional 6 h. The GFP signal (excitation, 488 nm; emission, 520 nm) was observed via a 63× oil objective on a Zeiss Imager confocal microscope. One hundred mitotic cells were analyzed for each experiment. Images were processed with ImageJ (NIH) and Photoshop (Adobe).

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