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Agrobacterium-Mediated Transformation of *Aspergillus awamori* in the Absence of Full-Length VirD2, VirC2, or VirE2 Leads to Insertion of Aberrant T-DNA Structures

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Reductions to 2, 5, and 42% of the wild-type transformation efficiency were found when *Agrobacterium* mutants carrying transposon insertions in *virD2*, *virC2*, and *virE2*, respectively, were used to transform *Aspergillus awamori*. The structures of the T-DNAs integrated into the host genome by these mutants were analyzed by Southern and sequence analyses. The T-DNAs of transformants obtained with the *virE2* mutant had left-border truncations, whereas those obtained with the *virD2* mutant had truncated right ends. From this analysis, it was concluded that the virulence proteins VirD2 and VirE2 are required for full-length T-DNA integration and that these proteins play a role in protecting the right and left T-DNA borders, respectively. Multicopy and truncated T-DNA structures were detected in the majority of the transformants obtained with the *virC2* mutant, indicating that VirC2 plays a role in correct T-DNA processing and is required for single-copy T-DNA integration.

Agrobacterium tumefaciens is capable of transferring a part of its tumor-inducing (Ti) plasmid (the transferred DNA, or T-DNA) to eukaryotic cells, where it stably integrates into the host genome. *A. tumefaciens* is now widely used for the transformation of plants, and more recently also for transformation of yeasts and fungi (3, 10). Proteins encoded by the virulence region of the Ti plasmid mediate T-DNA processing and transfer (13, 45). The VirD2 protein, in the presence of VirD1, recognizes the ends of the T-DNA, which is delimited by two T-DNA border repeats, and nicks the bottom strand of these 24-bp border repeats between the second and third bases. These nicks are then used as initiation and termination sites for the displacement of a linear single-stranded copy of the bottom strand of the T-DNA region, the T strand. The VirD2 protein stays covalently attached to the T strand via its tyrosine residue (Tyr29) (40). The right border is usually the initiation point for T-strand production due to the presence of a so-called overdrive located at that border. VirC1, one of the products of the *virC* locus, can bind this 25-bp overdrive sequence and thereby enhance T-strand production (32). The other product of the *virC* locus, the VirC2 protein, is also thought to play a role in efficient T-DNA transfer (43); however, its precise role in T-DNA formation is unknown. The T-strand–VirD2 complex is transported to the host via a pilus-like structure consisting of VirD4 and 11 different VirB proteins. Once in the host, the T strand is coated with the single-stranded DNA binding protein VirE2, which is transported to the host independently of the T strand (36). Binding of VirE2 to the T strand protects it against nucleolytic degradation and

turns the T strand into a transportable conformation, which facilitates the nuclear import of the T strand. The T complex, composed of the T strand, VirD2, and VirE2, is imported into the nucleus, where the T strand integrates stably into the host genome. Besides nicking the T-DNA borders, the VirD2 protein also plays a role in targeting the T strand to the host cell nucleus, due to a nuclear localization signal present in the C-terminal half of VirD2. T-DNA integration into the host genome is a random process, and it seems that the pathway of integration, homologous or nonhomologous, is mainly determined by host factors (3, 34).

In a recent paper, we have shown that, as for plants and yeasts, the *Agrobacterium* virulence proteins VirA, VirB, VirD1/VirD2, VirD4, and VirG are essential in order to obtain *Aspergillus awamori* transformants (19b). However, in the absence of the virulence proteins VirE2 and VirC2, the transformation frequency was reduced to 63 and 8% of the wild-type frequency. With the nonpolar 3' *virD2* *Agrobacterium* mutant, which lacks the C-terminal nuclear localization signal and omega domain of VirD2, a low transformation frequency (3%) for *A. awamori* was repeatedly found.

We were interested to find out whether this difference in transformation frequency would be reflected in changes in the structure of the integrated T-DNA. Precedents for this have been described. In plants, the absence of VirE2 leads to integration of 3'-truncated T-DNA copies (26) and a mutation in the C-terminal part of VirD2 leads to integration of 5'-truncated T-DNA copies (30). To determine the effect of 3' *virD2*, *virC2*, and *virE2* mutations in correct T-DNA integration in fungi, *A. awamori* was transformed with 3' *virD2*, *virE2*, and *virC2* *Agrobacterium* mutant strains and a number of transformants were subjected to analysis at the DNA level. T-DNA integration patterns of the transformants obtained with the *Agrobacterium* mutant strains were compared to integration patterns observed in transformants obtained with the wild-type

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Agrobacterium strain. Furthermore, upon T-DNA rescue from transformants with a single T-DNA integration, T-DNA borders and flanking genomic sequences were determined. Based on the T-DNA integration patterns, T-DNA borders, and genomic sequences observed, conclusions about the possible functions of the analyzed Vir proteins during *Agrobacterium*-mediated transformation of *A. awamori* could be made.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. *A. awamori* CBS115.52 (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) was transformed to hygromycin resistance using *Agrobacterium* strain LBA1100 (pAL1100ΔT-DNA Δ*tra* Δ*occ*) and the following transposon mutants: LBA1146 (pAL1100 [*virC2*::Tn3*HoHo1*]), LBA1147 (pAL1100 [*3' virD2*::Tn3*HoHo1*]), and LBA1149 (pAL1100 [*virE2*::Tn3*HoHo1*]) (1), carrying the binary vector pTAS10 (10). Introduction of pTAS10 into the *Agrobacterium* strains was carried out using electroporation as described by Mattanovich et al. (19a). *Escherichia coli* MC1061 (42) was used as a host for T-DNA rescue. *E. coli* and the *Agrobacterium* strains were grown overnight at 37 and 28°C in Luria-Bertani medium (27) containing antibiotics at the following concentrations: 50 μg of kanamycin/ml for *E. coli* and 250 μg of spectinomycin/ml, 75 μg of carbenicillin/ml, and 100 μg of kanamycin/ml for *A. tumefaciens*. The carbenicillin was omitted in cultures for the helper strain, LBA1100.

Agrobacterium-mediated transformation of *A. awamori* with *Agrobacterium* strains carrying the binary vector pTAS10 was performed as described by de Groot et al. (10) with minor adjustments (19b).

Chromosomal DNA isolation and Southern analysis. Filamentous-fungus chromosomal DNA was isolated as described by Kolar et al. (15). For Southern analysis, 0.5 to 1 μg of chromosomal DNA was digested with 10 U of BglII or HindIII and incubated overnight at 37°C. The samples were loaded on a 1% 0.5× Tris-borate-EDTA gel and run for 18 h at 50 V. The digested DNA was transferred to Hybond-N+ (Amersham Pharmacia) under vacuum (VacuGene XL; Amersham Pharmacia) as described by the manufacturer.

The probes used for Southern analysis were a 3.1-kb XhoI-HindIII fragment of plasmid pAN7.1 (24), corresponding to the hygromycin cassette, and linearized pUC9 (38). For the detection of non-T-DNA (binary vector backbone), a linearized pSDM14 (23) plasmid was used. The Rediprime II DNA labeling system (Amersham Pharmacia) was used to label the probes with [α -³²P]dCTP. Hybridization was carried out with GeneScreen (DuPont NEN Research Products) as described by the manufacturer. Southern hybridization signals were detected using a PhosphorImager (Molecular Dynamics).

Plasmid rescue and sequencing. Filamentous-fungus chromosomal DNA (1 μg) was digested with 10 U of BglII. After an overnight incubation at 37°C, the restriction enzyme was removed by phenol-chloroform extraction, followed by precipitation of the DNA. Digested DNA (500 ng) was subsequently used for ligation, which was performed overnight at 16°C with 2 U of T4 DNA ligase (Gibco BRL). One-fifth of the ligation mixture was electroporated (2.5 kV; 100 Ω; 25 μF) (Gene Pulser; Bio-Rad) to *E. coli* MC1061. Plasmid DNA was isolated as described by Sambrook et al. (27).

Sequencing was carried out on a Perkin-Elmer ABI PRISM 310 using the PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and the following primers: for the right border, pAwK08 (5'-GGAGAT GTTGCTGAAGTC-3') or pGPD2 (5'-ACTGCCGGTGATTTCGATGAA-3'); for the left border, pIC20R (5'-GGCACCCAGGCTTTACAC-3') or pIC20-6 (5'-TGAGCTATGAGAAAGCGCCA-3').

RESULTS

Analysis of T-DNA integration patterns. We previously showed that transformation of *A. awamori* with *Agrobacterium* strains carrying a transposon insertion in 3' *virD2*, *virC2*, and *virE2* led to a reduction of 97, 92, and 37%, respectively, compared to the wild-type *Agrobacterium* strain (19b). In this study, we were interested in the T-DNA integration patterns of the transformants obtained with the *Agrobacterium* mutant strains. To allow reisolation of the T-DNA after integration using a T-DNA rescue approach (see Materials and Methods), the *Agrobacterium* strains were transformed with pTAS10,

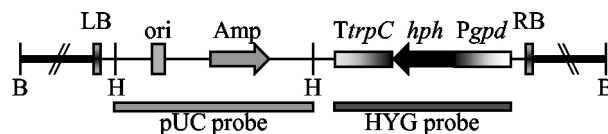


FIG. 1. T-DNA of pTAS10. The thin bar represents T-DNA and the thick bars represent chromosomal DNA. LB, left border; RB, right border; ori, pUC origin of replication; Amp, gene conferring carbenicillin and ampicillin resistance in *E. coli*; Pgd, *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter; hph, hygromycin B resistance gene; TrpC, *A. nidulans* trpC terminator; B, BglII restriction site; H, HindIII restriction site.

which contains the complete pUC9 plasmid, in addition to the hygromycin selection marker (10). Transformation of *A. awamori* with the *Agrobacterium* mutants carrying pTAS10 resulted in reduced transformation frequencies of 98, 95, and 58% for 3' *virD2*, *virC2*, and *virE2*, respectively, which is comparable to our earlier results (see above).

For Southern blot analysis, total DNA was isolated and digested with BglII, which does not cut in the T-DNA, or with HindIII, which cuts twice in the T-DNA. To distinguish among single, tandem-repeat, and inverted-repeat T-DNA integrations, different probes were used (Fig. 1). Deletion of ≥ 76 nucleotides at the left T-DNA border could be detected due to loss of the HindIII restriction site located at the left border (Fig. 1). The presence of non-T-DNA vector sequences was determined by hybridizing the blots with a linear binary vector, pSDM14, which corresponds to the pBIN19 sequence present in the backbone of pTAS10.

Three main integration patterns were observed in transformants obtained with the wild-type *Agrobacterium* strain. A single T-DNA integration was found in 62.5% (10 of 16) of the transformants analyzed (Fig. 2A, lanes 1, 4, 7, and 10), whereas in 12.5% (2 of 16), a tandem T-DNA integration consisting of two T-DNAs was observed (Fig. 2A, lanes 2, 5, 8, and 11, and Table 1). In the remaining four transformants, it seemed that two T-DNAs integrated at random on different loci (Fig. 2A, lanes 3, 6, 9, and 12). However, further analysis revealed that vector sequences located outside of the T-DNA might be present in these transformants (Fig. 2A, lane 18; a 4-kb fragment). No strong signal was observed in the lane with BglII-digested DNA, indicating that the entire vector is not present (Fig. 2A, lane 15). Based on plasmid rescue and border sequence analyses, it was found that in this transformant a rearrangement of the binary vector had occurred. Part of the binary vector and left border was present, but in reverse orientation (data not shown). None of the other transformants analyzed (12 of 16) contained non-T-DNA vector sequences.

Analysis of T-DNA–fungal-DNA junctions in transformants containing a single T-DNA copy revealed that in all of those analyzed (eight of eight), 1 or 2 nucleotides adjacent to the right border nick sites in the binary vector were preserved in the T-DNA (Fig. 3), as observed in plants (12, 35). In one transformant, 2 nucleotides were present outside the VirD1/VirD2 nick area (Fig. 3). The left T-DNA ends were spread over the 24-bp repeat in all transformants analyzed, similar to what was observed in plants (12).

Role of VirD2 in T-DNA integration. Transformants obtained with the *Agrobacterium* 3' *virD2* mutant strain displayed

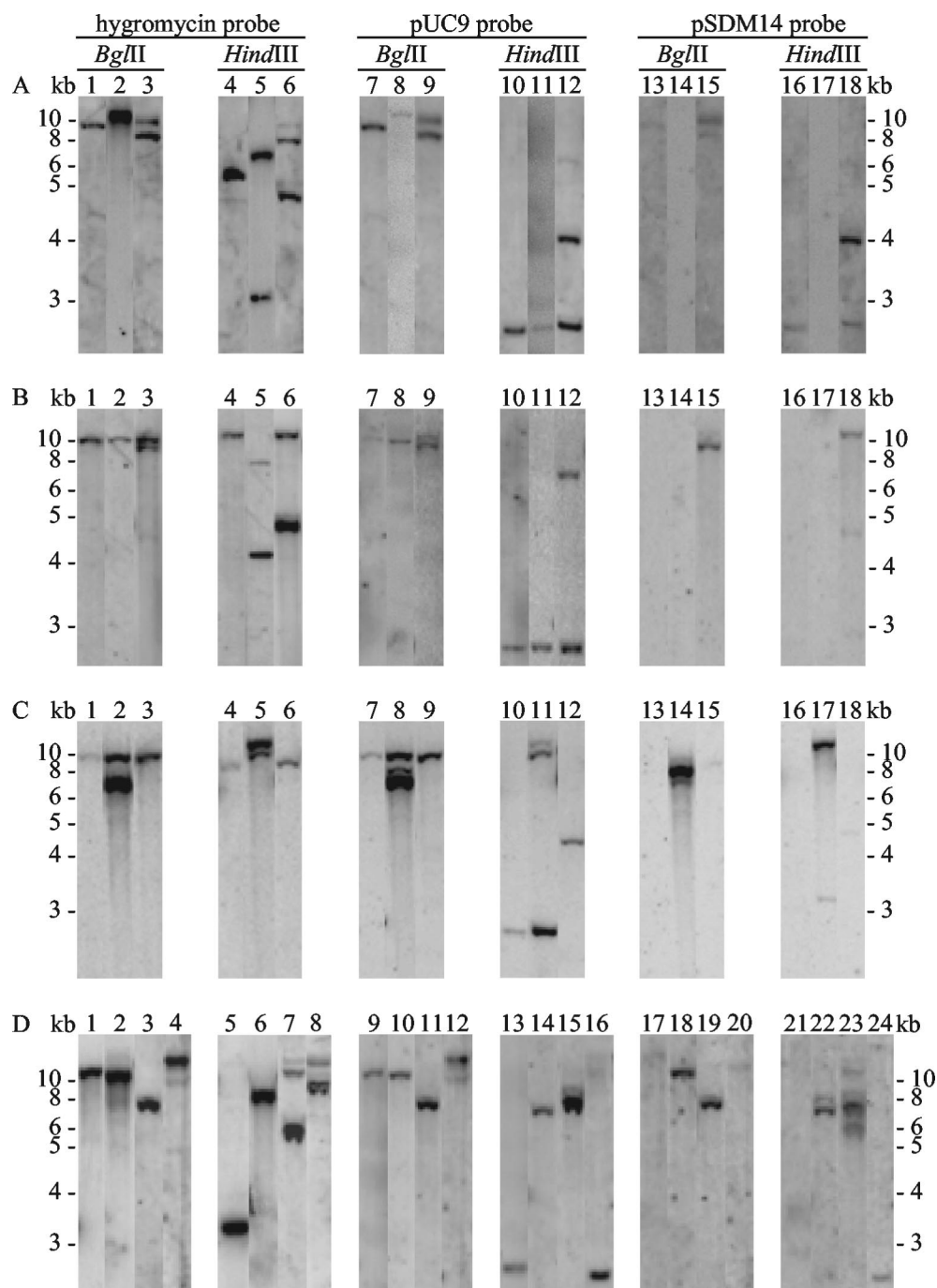


FIG. 2. Southern blot analysis of transformants obtained with wild-type (A), 3' *virD2* mutant (B), *virE2* mutant (C), and *virC2* mutant (D) *Agrobacterium* strains. Chromosomal DNA was digested with *Bgl*II or *Hind*III, and the fragments were separated on gels, blotted, and hybridized with hygromycin, pUC9, and pSDM14 probes. This figure is composed from different blots, which results in minor differences in fragment sizes. (A) Lanes 1, 4, 7, 10, 13, and 16, [1100-pTAS10]#9; lanes 2, 5, 8, 11, 14, and 17, [1100-pTAS10]#1; lanes 3, 6, 9, 12, 15, and 18, [1100-pTAS10]#10. (B) Lanes 1, 4, 7, 10, 13, and 16, [1147-pTAS10]#8; lanes 2, 5, 8, 11, 14, and 17, [1147-pTAS10]#11; lanes 3, 6, 9, 12, 15, and 18, [1147-pTAS10]#5. (C) Lanes 1, 4, 7, 10, 13, and 16, [1149-pTAS10]#7; lanes 2, 5, 8, 11, 14, and 17, [1149-pTAS10]#8; lanes 3, 6, 9, 12, 15, and 18, [1149-pTAS10]#10. (D) Lanes 1, 5, 9, 13, 17, and 21, [1146-pTAS10]#11; lanes 2, 6, 10, 14, 18, and 22, [1146-pTAS10]#15; lanes 3, 7, 11, 15, 19, and 23, [1146-pTAS10]#8; lanes 4, 8, 12, 16, 20, and 24, [1146-pTAS10]#10. The weak signal present in lanes A13, A15, A16, A18, C18, and D24 is due to incomplete stripping of the blots.

TABLE 1. T-DNA integration patterns observed in transformants obtained with wild-type and mutant *Agrobacterium* strains

Integration pattern ^a	No. of transformants with:			
	Wild type ^b	3' <i>virD2</i> mutant	<i>virE2</i> mutant	<i>virC2</i> mutant
1× T-DNA	10	8	4	6
1× T-DNA – LB			8	
2× T-DNA	2	1		1
1× T-DNA + backbone	1 tr		1	2
2× T-DNA + backbone	3 tr	1	2	
1× T-DNA + multiple backbone				3
Multiple T-DNA				2
Multiple T-DNA + multiple backbone				2
No. of transformants analyzed	16	10	15	16

^a LB, left border.
^b tr, truncated.

integration patterns similar to those observed in transformants obtained with the wild-type *Agrobacterium* strain. Single (80%) and double (10%) T-DNA integrations were found (Table 1 and Fig. 2B, lanes 1 and 4 and lanes 2 and 5). In one of the transformants, a complete binary vector was found (Fig. 2A, lanes 15 and 18). In that transformant, two T-DNAs were present, together with the entire backbone sequence. Analysis of the T-DNA borders of single-copy transformants revealed that in two of the five transformants analyzed a truncation of the right border was observed (Fig. 3). The left T-DNA ends were spread over the 24-bp repeat, except in one transformant, in which a deletion of 38 bp was observed (Fig. 3).

Role of VirE2 in T-DNA integration. Also, for transformants obtained with the *Agrobacterium virE2* mutant strain, mainly single T-DNA integrations were observed (Table 1 and Fig. 2C, lanes 1 and 4). In 3 out of the 15 transformants analyzed, T-DNA backbone sequences were detected, either as a result of a read-through of the left border or as a start of T-DNA

synthesis at the left border (Table 1 and Fig. 2C, lanes 2, 5, 14, and 17). However, in >50% (8 of 15) of the transformants analyzed, truncation of the left border was observed (Table 1). This was confirmed by T-DNA border analysis for two single-copy transformants in which large deletions of 100 to 300 nucleotides were found (Fig. 3). The right T-DNA border–fungal-DNA junctions resemble those observed in transformants obtained with the wild-type *Agrobacterium* strain (Fig. 3).

Role of VirC2 in T-DNA integration. Transformants obtained with the *Agrobacterium virC2* mutant displayed aberrant integration patterns which were not observed in other transformants. A relatively small number of the transformants (6 of 16) had a single T-DNA integration (Table 1 and Fig. 2D, lanes 1, 5, 9, and 13). The majority of the transformants analyzed had multiple T-DNA integrations with or without backbone sequences. In one case, more than one copy of the binary vector could be detected. In addition to single T-DNA integrations (Fig. 2D, lanes 1, 5, 9, 13, 17, and 21), five different integration patterns could be distinguished. One transformant had a double T-DNA integration, and two transformants had a single T-DNA integration with backbone sequences (Table 1; Southern data not shown). Such single and double T-DNA integrations were also observed in transformants obtained with the wild-type *Agrobacterium* strain. However, the following three integration patterns were observed only in transformants obtained with the *virC2* *Agrobacterium* mutant strain. First, a single T-DNA integration was found accompanied by truncated binary-vector backbone sequences (Table 1 and Fig. 2D, lanes 2, 6, 10, 14, 18, and 22). The other two patterns that were observed were multiple T-DNA integrations with or without multiple backbone sequences (Table 1 and Fig. 2D, lanes 3, 7, 11, 15, 19, and 23 and lanes 4, 8, 12, 16, 20, and 24, respectively).

Sequence analysis of T-DNA–fungal-DNA junctions in two single-copy T-DNA transformants revealed that the T-DNA

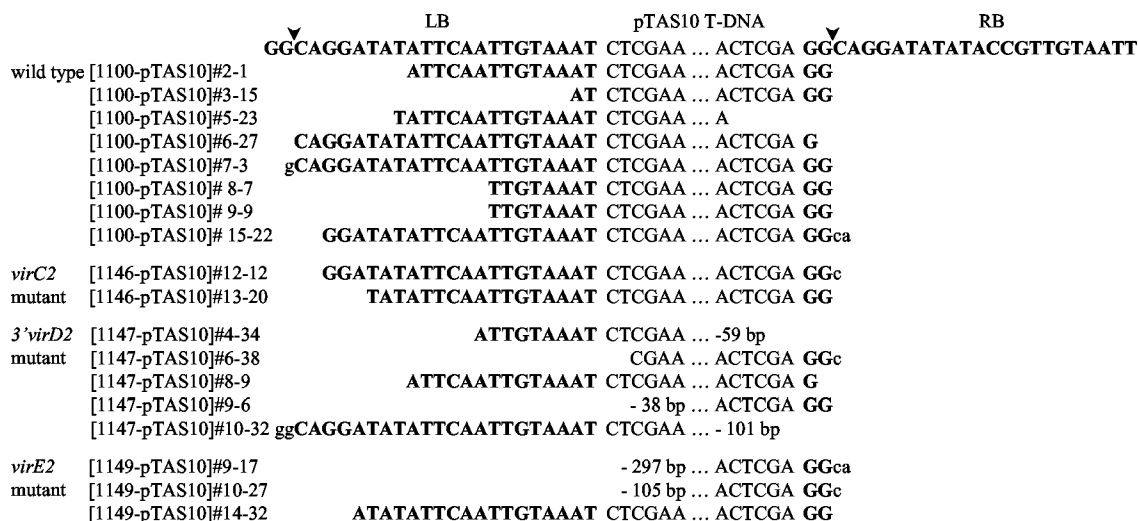


FIG. 3. Sequences of T-DNA borders in *A. awamori* transformants obtained with wild-type and mutant *Agrobacterium* strains. All sequences are shown as 5'→3'. Uppercase letters represent sequences originating from the T-DNA, boldface uppercase letters represent the 24-bp T-DNA border repeats, and lowercase letters correspond to genomic DNA or leftover border sequences; the arrowheads mark border nick sites. LB, left border; RB, right border.

ends resembled those observed in transformants obtained with the wild-type *Agrobacterium* strain (Fig. 3).

From these results, it can be concluded that transformants obtained with the wild-type and the mutant 3' *virD2* and *virE2* *Agrobacterium* strains showed predominantly single and random T-DNA integration and that based on the sequences of T-DNA–fungal-DNA junctions, truncation of the right and left borders was found in a number of transformants obtained with the 3' *virD2* and *virE2* *Agrobacterium* mutants, respectively. In transformants obtained with the *virC2* *Agrobacterium* mutant strain, multiple T-DNA integrations linked to non-T-DNA sequences were found. These integration patterns were not observed in transformants obtained with the wild-type strain.

Analysis of flanking fungal DNA. In addition to analysis of the border sequences, a homology search of the flanking fungal DNA was performed with the BLASTN and BLASTX programs (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the nonredundant databases. Two out of the 19 transformants analyzed showed possible homology to the database (E value < $1e^{-4}$). Transformant [1100-pTAS10]#9 had a T-DNA integration in a putative karyopherin-encoding gene, while in transformant [1146-pTAS10]#13, the T-DNA had integrated close to a putative phosphoglucomutase-encoding gene. The remainder of the transformants had a T-DNA integration in a site that showed no significant homology (E value > $1e^{-4}$). The isolated chromosomal border sequences were also compared to the *A. fumigatus* database of The Institute for Genomic Research (<http://www.tigr.org/tdb/fungal/>; search performed on 6 August 2003). In transformant [1100-pTAS10]#9, in which the T-DNA had integrated into a putative karyopherin-encoding gene, hits at the same contig were found for the left and right border sequences. A gap of 11 nucleotides was present between the left and right border sequences compared to the contig, indicating that a deletion of 11 nucleotides had occurred at the T-DNA integration site. In transformant [1146-pTAS10]#13, in which the left border of the T-DNA had integrated close to a putative phosphoglucomutase-encoding gene, the corresponding *A. fumigatus* homolog was also identified. The sequence of the right border of the [1146-pTAS10]#13 strain did not show homology to the *A. fumigatus* genome. Comparison of the sequences beyond the borders of the other transformants to the *A. fumigatus* genome sequence using BLASTN identified two further integrations, which had E values of < $1e^{-6}$. The integration site at the left border of transformant [1100-pTAS10]#5 showed homology (E value < $1e^{-11}$) to four contigs, but these regions encode unknown proteins. The sequence of the right border of transformant [1147-pTAS10]#9 showed homology (E value < $1e^{-6}$) to contig 2054. Further analysis revealed that the insertion had occurred close to a gene that shows homology to a gypsy-like retrotransposon-encoding gene. Sequence comparison of the integration sites of the other transformants showed E values of $1e^{-4}$ or higher, and they were not considered relevant. Although *A. awamori* and *A. fumigatus* are related species, major differences might exist in their noncoding regions. This could explain the low number of hits that were found when *A. awamori* DNA was compared to *A. fumigatus* DNA. It is likely that only when the T-DNA integrates close to or into an open reading frame can an extensive homology to the *A. fumigatus*

database be found. No apparent homology was present among the different integration sites.

The variation of the T-DNA border sequences and the flanking chromosomal DNAs observed in all of the transformants analyzed resembles the variation observed in plants and yeasts after random T-DNA integration.

DISCUSSION

A. tumefaciens, widely used for the transformation of plants and more recently also for yeasts and fungi, is capable of transferring its T-DNA to the host, where it integrates into the host genome via homologous or nonhomologous recombination. A set of virulence proteins, located on the tumor-inducing plasmid of *A. tumefaciens*, are involved in the generation of the T strand and its subsequent transfer to the host. Different requirements for the presence of the virulence proteins for optimal T-DNA transfer among plants, yeasts, and fungi were observed (19b). To elucidate the effects and to infer the roles of the virulence proteins VirD2, VirE2, and VirC2 in T-DNA transfer and integration into filamentous fungi, the fate of the T-DNA integrated into the genome of *A. awamori* was determined.

The molecular analysis of *A. awamori* transformants obtained with the wild-type *Agrobacterium* strain revealed that T-DNA integration resembles that observed in plants and yeasts. Integration of the T-DNA occurred predominantly as a single copy, but inverted or tandem integration of the T-DNA was also seen in a minority of the transformants analyzed, as observed in plants, yeasts, and fungi (4, 7, 9, 25). Also, the presence of non-T-DNA vector sequences, as in plants and fungi, was observed in 25% of the *A. awamori* transformants analyzed. The transfer of non-T-DNA vector sequences linked to the T-DNA has also been observed in *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Nicotiana tabacum* at frequencies of 50, 60, 62, and 75%, respectively (4, 7, 16, 41). Sequence analysis of the *A. awamori* transformants obtained with the wild-type *Agrobacterium* strain revealed that the right end of the T-DNA clustered mainly around the first 2 nucleotides of the 24-bp right border repeat. At the 24-bp left border repeat, more variation was observed, but no large deletions, except in the transformant in which an inverted part of the binary vector had cointegrated with the T-DNA. The observed variation at the right and left borders is in line with observations made in plants, yeasts, and fungi (3, 10, 12, 19, 21). Analysis of the genomic flanking regions confirmed that the T-DNA had integrated at random, as observed in the Southern analysis, because no large homologies were found between the T-DNA integration sites. Furthermore, comparison of the integration sites to the *A. fumigatus* database revealed that in two of the transformants analyzed, a T-DNA insertion close to or into an open reading frame was found. Although the transformants displayed no aberrant phenotype, this shows that T-DNA can be used effectively as a gene tag in filamentous fungi, in addition to transposon mutagenesis, which is developed for various fungi (14, 18, 39). The sequence data of the T-DNA rescues suggest that the T-DNA had integrated at random and via nonhomologous recombination, since no extensive homologies were found between the T-DNA and the integration site or among the integration sites themselves.

In transformants obtained with the *virE2* and 3' *virD2* *Agrobacterium* mutants, mainly single T-DNA integrations were found, as described above for the transformants obtained with the wild-type *Agrobacterium* strain. However, in >50% of the transformants analyzed that were obtained with the *virE2* *Agrobacterium* mutant, T-DNA truncation of ≥ 76 nucleotides at the left border was observed. T-DNA rescues of two of these transformants revealed that, in agreement with the Southern analysis, deletions of 100 and 300 nucleotides had occurred. Virulence protein E2, a single-stranded DNA binding protein, performs many functions during the *Agrobacterium* infection process, including protection of the T strand against nucleolytic degradation, facilitation of T-DNA transport through the nuclear pore, and nuclear targeting via interaction with cytoplasmic chaperones (5, 33). Inactivation of VirE2 might make the T-DNA more vulnerable to nucleolytic degradation, resulting in a lower transformation frequency, as observed in the *A. awamori* transformation, and to integration of truncated T-DNA, as observed in 50% of the transformants analyzed. Truncation of the left T-DNA border has also been observed in transgenic plants obtained with an *Agrobacterium virE2* mutant (9, 12, 26).

T-DNA rescues of five transformants obtained with the 3' *virD2* *Agrobacterium* mutant revealed that in two transformants a deletion of >60 nucleotides at the right T-DNA border had occurred. The VirD2 protein of this *Agrobacterium* mutant lacks 73 C-terminal amino acids, thereby deleting the nuclear localization signal and the omega sequence. The *Agrobacterium* VirD2-T-DNA complex is translocated into host cells through the VirB/VirD4 type IV secretion system. In addition, the *Agrobacterium* effector proteins VirE2, VirE3, and VirF, which are also translocated into the host through the VirB/VirD4 type IV secretion system, have been shown to contain a C-terminal transport signal that is sufficient for translocation into host cells (36, 37). It has been suggested that the C-terminal domain of VirD2 may contain a translocation signal. However, our data show that the truncated VirD2 protein, containing a C-terminal deletion of 73 amino acids, is still transferred with the attached T strand into *A. awamori* at low efficiency, indicating that there could be another (cryptic) transport domain present in the truncated VirD2 protein or that another protein, for example, VirD1, binds to VirD2 and carries the complex to the host. Limited T-DNA transfers to plants from the same *virD2* mutant used in this study and from mutants with (smaller) deletions in the C terminus have been described (17, 28).

The nuclear localization signal plays an important role in T-DNA targeting to the nucleus. Deletion of the nuclear localization signal leads to reduced or abolished T-DNA transfer and integration (3, 11). For the omega domain, controversial functions had been reported. It was stated that the omega domain does not play a role in T-DNA transfer and nuclear targeting, but a role in efficient T-DNA integration could not be ruled out (2, 22). Precise VirD2 Δ NLS and VirD2 Δ Ω *Agrobacterium* mutants assayed on *A. awamori* resulted in a lower transformation frequency than the wild type (C. B. Michielse, unpublished results), as observed for plants (2, 22, 28, 29). Based on Southern analysis, the right T-DNA border was preserved in the transformants obtained with these precise deletion mutants (Michielse, unpublished), indicating that the

absence of the nuclear localization signal or the omega domain in the 3' *virD2* *Agrobacterium* mutant does not explain the observed truncation of the right T-DNA border in the transformants obtained with the mutant. It could be that the observed truncation of the right border in *A. awamori* transformants is due to an incorrect conformation of the VirD2 protein, affecting its function in nuclear targeting and protection of the right border via its binding to the 5' end of the T-DNA, or there could be an unidentified factor located in the 73 deleted amino acids that could be involved in T-DNA transfer and/or integration.

Mutations in VirC1 or VirC2 led to reduced virulence of *A. tumefaciens* (6). In earlier studies, it was shown that VirC1 can bind to the overdrive sequence located adjacent to the right-border repeat of the T region (31, 32). Furthermore, both *virC1* and *virC2* mutants showed reduced border cleavage (32), suggesting that both VirC1 and VirC2 are involved in T-DNA processing and T-strand formation. In plant tissues transformed by *Agrobacterium virC* mutants, there are many fewer T-DNA transcripts (transiently) present than in those transformed by the wild-type strain, indicating reduced T-DNA transfer (20). A role for VirC2 in the generation of a T-DNA transfer intermediate is in agreement with the observed 13-fold reduction in the number of transformants in *Agrobacterium*-mediated transformation of *A. awamori* (Michielse et al., submitted) and *S. cerevisiae* (A. den Dulk-Ras, C. B. Michielse, and P. J. J. Hooykaas, unpublished data). If VirC2 in fact has a role in T-DNA border processing, this might be reflected in the T-DNA structure present in transformed eukaryotic cells. In this paper, we provide for the first time further evidence for a possible role of VirC2 in T-DNA processing through the detailed analysis of the T-DNA structures present in *A. awamori* transformants obtained with a *virC2* *Agrobacterium* mutant. Southern analysis of transformants revealed the presence of aberrant T-DNA integration patterns in 10 out of the 16 transformants analyzed. Multicopy T-DNA integration and integration of non-T-DNA vector sequences were observed for the majority of transformants. Such structures can be explained by poor border nicking and border skipping and are in line with a role for VirC2 in T-DNA border processing. It will be of interest to study the effect on T-DNA border processing in plant transformants obtained with a *virC2* *Agrobacterium* mutant to confirm its role in T-DNA processing. The molecular mechanism by which VirC2 might assist in the generation and/or processing of a T strand is not known. The *virC* operon consists of two genes, those for VirC1 and VirC2. VirC1 has been shown to bind to the overdrive sequence to enhance T-DNA border nicking (31, 32) either by recruiting the VirD1/VirD2 complex to the T-DNA borders or by facilitating the binding of the VirD1/VirD2 complex to the borders. Our hypothesis is that VirC2 is somehow connected to the function of VirC1 in recruiting or facilitating the binding of the VirD1/VirD2 complex to the borders. However, the exact role of VirC2 during T-DNA processing, which could explain the observed aberrant T-DNA structures, remains to be resolved by *in vitro* biochemical experiments.

Thus, based on Southern blot analysis and T-DNA rescue, it can be concluded that T-DNA integration into the fungal genome occurs via nonhomologous recombination and that single and double T-DNA integrations occur with frequencies

comparable to those observed in plants (8, 44). Furthermore, no major differences in T-DNA integration patterns were observed between transformants obtained with 3' *virD2* and *virE2* *Agrobacterium* mutant strains and the transformants obtained with the wild-type *Agrobacterium* strain. However, based on the sequences of T-DNA–fungal-DNA junctions, truncation of the right and left borders was found in a larger number of transformants obtained with the *virD2* and *virE2* *Agrobacterium* mutants, respectively, indicating a protective role for these virulence proteins during T-DNA transfer and integration. Inactivation of VirC2 led to aberrant and multicopy T-DNA integration, indicating that VirC2 might be involved in correct T-DNA border processing.

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