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Role of bacterial virulence proteins in *Agrobacterium*-mediated transformation of *Aspergillus awamori*

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Abstract

The *Agrobacterium*-mediated transformation of *Aspergillus awamori* was optimized using defined co-cultivation conditions, which resulted in a reproducible and efficient transformation system. Optimal co-cultivation conditions were used to study the role of *Agrobacterium tumefaciens* virulence proteins in T-DNA transfer. This study revealed that inactivation of either of the regulatory proteins (VirA, VirG), any of the transport pore proteins (VirB), proteins involved in generation of the T-strand (VirD, VirC) or T-strand protection and targeting (VirE2) abolishes or severely reduces the formation of transformants. The results indicate that the *Agrobacterium*-mediated transformation of *A. awamori* requires an intact T-DNA machinery for efficient transformation; however, the plant host range factors, like VirE3, VirH, and VirF, are not important.

Index descriptors: Agrobacterium tumefaciens; T-DNA; Virulence proteins; DNA-transformation; *Aspergillus awamori*; Filamentous fungi

1. Introduction

Many fungal species can be transformed by $CaCl_2$/PEG-mediated transformation. This method mainly depends on the success of viable protoplast preparation (Goosen et al., 1991). The main bottleneck for the generation of protoplasts is the variation between different enzyme batches in activity of digestive enzymes (Fincham, 1989; Ruiz-Diez, 2002), which are used to remove the fungal cell wall. Therefore, cell wall digestion must be monitored in time and optimal conditions have to be found for each fungus and enzyme batch to achieve enough viable protoplasts.

To avoid the preparation of protoplasts other methods have been developed for the transformation of fungi, like lithium acetate treatment, particle bombardment, and electroporation (reviewed by Hynes, 1996; Ruiz-Diez, 2002). However, these methods can only be applied to a limited number of fungi and usually result in a low transformation frequency (Dhawale et al., 1984; Kapoor, 1995; Mellon et al., 1987; Ozeki et al., 1994).

Recently, a new methodology for fungal transformation was introduced, derived from the successful plant transformation system based on the soil bacterium, *Agrobacterium tumefaciens* (de Groot et al., 1998). This bacterium can equally efficiently transform protoplasts and fungal spores (de Groot et al., 1998). Furthermore, the *Agrobacterium*-mediated gene delivery system appears to be a more efficient transformation method than the $CaCl_2$/PEG-mediated transformation (de Groot et al., 1998). Analysis of the transformants obtained by the *Agrobacterium*-mediated gene transfer system showed that foreign DNA integrated at a random position in the genome and predominantly as a single copy. The *Agrobacterium* system has since been successfully used to transform spores, mycelium, and even fruiting body tissue of a large number of different fungi, e.g., *Coccidioidis immitis*, *Fusarium oxysporum*, *Magnaporthe grisea*, and *Agaricus bisporus* (Abuodeh et al., 2000; Chen et al., 2000; Mullins et al., 2001; Rho et al., 2001).

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Agrobacterium tumefaciens, a plant pathogen, is capable of transforming plants, yeasts, and fungi by introducing a piece of DNA (transferred DNA or T-DNA) located between two 24-bp border repeats on the binary vector of the bacterium into the nuclear genome of these organisms. Virulence proteins, which are encoded by the virulence region located on the tumour inducing plasmid (Ti-plasmid), are essential for T-DNA transfer. The virulence proteins VirA and VirG form a two-component regulatory system, which is activated upon recognition of specific phenolic compounds and then activates the entire T-DNA transfer machinery. The products of the *virC* and *virD* operons generate a single stranded T-DNA copy, which is transported as a VirD2–T-strand complex to the host cytoplasm via a type IV secretion system (VirB1–11 and VirD4). Once in the host cytoplasm the T-strand will be translocated to the host nucleus due to nuclear localization signals present in VirD2 and VirE2 followed by integration into the host genome (reviewed by Gelvin, 2000; Zupan et al., 2000).

Previously it was found that *A. tumefaciens* uses its virulence system to transform fungi (de Groot et al., 1998). For a limited number of virulence genes it has been determined to which extend these virulence genes are required for *Agrobacterium*-mediated transformation of the yeast *Saccharomyces cerevisiae* (Bundock et al., 1995). In this study we have analyzed the role of an extended number of virulence genes and determined which Vir functions are necessary for T-DNA transfer to the filamentous fungus *Aspergillus awamori*. Our results reveal many similarities, but also some interesting differences in the requirements between plant, yeast, and fungal transformation by *A. tumefaciens*.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

Aspergillus awamori CBS115.52 (CBS, The Netherlands) was transformed to hygromycin resistance using *Agrobacterium* strains listed in Table 1 carrying the binary vector pUR5750 (de Groot et al., 1998). Introduction of pUR5750 into the *Agrobacterium* strains was carried out via electroporation as described by Mattanovich et al. (1989). *Agrobacterium* strains were grown overnight at 28 °C in LB medium (Sambrook et al., 1989) containing the appropriate antibiotics (Table 1) at the following concentrations: 20 μg/ml rifampicin, 250 μg/ml spectinomycin, 75 μg/ml carbenicillin, and 100 μg/ml kanamycin.

Statistical analysis (independent Student's *t* test) was performed with Microcal Origin 5.0.

2.2. Agrobacterium-mediated transformation of A. awamori

Co-cultivation of *A. awamori* and *Agrobacterium* strains carrying the binary vector pUR5750 was performed as described by de Groot et al. (1998) with minor adjustments. *A. awamori* conidia were obtained after growing the strain on complete medium (Punt and van den Hondel, 1992) for four days at 30 °C. Subsequently, conidia were isolated with physiological salt and used directly for transformation. *Agrobacterium* strains carrying pUR5750 were grown overnight in LB containing the appropriate antibiotics. An aliquot (1.5 ml) of the overnight culture was centrifuged at 16,060*g*. The pellet was washed once with 200 μl induction medium (Bun-

Table 1
Agrobacterium strains used in this study

Strain	Chromosomal background	Resistance	Plasmid
LBA1100[a]	C58	rif, spc	pAL1100ΔT-DNA, Δ*tra*, Δ*occ*
LBA1141[a]	C58	rif, spc, cb	pAL1100(*virH*::Tn3*HoHo1*)
LBA1142[a]	C58	rif, spc, cb	pAL1100(*virA*::Tn3*HoHo1*)
LBA1143[a]	C58	rif, spc, cb	pAL1100(*virB4*::Tn3*HoHo1*)
LBA1144[a]	C58	rif, spc, cb	pAL1100(*virB7*::Tn3*HoHo1*)
LBA1145[a]	C58	rif, spc, cb	pAL1100(*virG*::Tn3*HoHo1*)
LBA1146[a]	C58	rif, spc, cb	pAL1100(*virC2*::Tn3*HoHo1*)
LBA1147[a]	C58	rif, spc, cb	pAL1100(3′*virD2*::Tn3*HoHo1*)
LBA1148[a]	C58	rif, spc, cb	pAL1100(*virD4*::Tn3*HoHo1*)
LBA1149[a]	C58	rif, spc, cb	pAL1100(*virE2*::Tn3*HoHo1*)
LBA1150[a]	C58	rif, spc, cb	pAL1100(*virD1*::Tn3*HoHo1*)
LBA1151[a]	C58	rif, spc, cb	pAL1100(5′*virD2*::Tn3*HoHo1*)
LBA2561[b]	C58	rif, spc	pAL1100Δ*virF*
LBA2565[c]	C58	rif, spc	pAL1100Δ*virE3*
A348[d]	C58	rif	pTiA6NC
A348[d]	C58	rif	pTiA6NCΔ*virB*X[e]

[a] Beijersbergen et al. (1992).
[b] Schrammeijer et al. (1998).
[c] Schrammeijer et al. (2003).
[d] Berger and Christie (1994).
[e] X represents the deleted *virB* gene, numbered from 1 to 11 (rif, rifampicin; spc, spectinomycin; cb, carbenicillin).

dock et al., 1995) and resuspended in 5 ml IM with or without 200 μM acetosyringone (AS). After incubation for 5–6 h at 28 °C on a rotary shaker (200 rpm) the optical density at 600 nm was measured and if necessary the culture was diluted to $OD_{600} \sim 0.8$ ($4–5 \times 10^8$ bacterial cells/ml).

An aliquot of 100 μl spore-stock solution with a concentration of 10^7 spores/ml was mixed with 100 μl of the induced *Agrobacterium* culture. The mixture was spread out on nitrocellulose (Hybond-N, Amersham–Pharmacia), which was placed on IM containing agar plates and incubated at different temperatures as indicated in Section 3. After co-cultivation the filters were transferred to agar plates containing minimal medium (Punt and van den Hondel, 1992) with 100 μg/ml hygromycin and 200 μM cefotaxim, followed by an incubation of four days at 30 °C.

3. Results

3.1. Optimization of A. tumefaciens-mediated transformation of A. awamori

In initial transformation experiments of *A. awamori* using the *Agrobacterium* system variations in the transformation frequencies between different experiments were observed. To improve the reproducibility and efficiency of the transformation system, different co-cultivation conditions were compared. From experiments with plants it is known that both the temperature and length of the co-cultivation period are important factors affecting the capacity of *A. tumefaciens* to transfer T-DNA to its host (Dillen et al., 2002; Fullner and Nester, 1996; Kurdirka et al., 1986; Salas et al., 2002). Therefore, co-cultivation of *A. awamori* and *A. tumefaciens* was performed at controlled and fixed temperatures and for different incubation periods. Mixtures of *A. tumefaciens* and spores were incubated on agar plates containing induction medium for one to three days at different temperatures (20, 22.5, 25, and 28 °C) before transferring the filters to agar plates containing selective medium. *A. awamori* was transformed with two different *Agrobacterium* strains, A348 and LBA1100, both carrying the binary vector pUR5750, which contains an *Escherichia coli* hygromycin resistance gene under control of an *Aspergillus nidulans gpd* promoter and *trpC* terminator between left and right border (de Groot et al., 1998). The transformation frequency, based on the number of hygromycin resistant colonies per 10^6 spores, was determined for each co-cultivation condition.

For *Agrobacterium* strain LBA1100, the most reproducible transformation frequencies without statistical differences (independent Student's *t* test, significance level 0.05) were obtained when co-cultivations were done for 2 or 3 days at 22.5 or 25 °C (Table 2A). Co-cultivation at 20 °C resulted in variable transformation frequencies, which hampered a meaningful statistical analysis. Co-cultivation at 28 °C resulted in a high background growth of *A. awamori* and a decrease in the transformation frequency. Statistical analysis showed that co-cultivation for 2 or 3 days at 22.5 or 25 °C was significant better to a 1 day co-cultivation period at either temperature.

Table 2
Optimization of T-DNA transfer of (A) LBA1100 and (B) A348 by variation of co-cultivation period and temperature

Co-cultivation condition	20 °C	22.5 °C	25 °C	28 °C
(A)				
1 day	1 ± 1[a]	11 ± 10	50 ± 22	49 ± 16
2 days	98 ± 74	158 ± 52	175 ± 35	135 ± 92
3 days	109 ± 129	183 ± 14	167 ± 58	88[b] ± 124
(B)				
1 day	0 ± 1[a]	0 ± 1	12 ± 4	10 ± 6
2 days	27 ± 4	101 ± 98	158 ± 38	103 ± 38
3 days	113 ± 32	130 ± 55	145[b] ± 64	67[b] ± 115

[a] Values represent the mean of three independent experiments and indicate the number of transformants per 10^6 spores.
[b] High background growth of *A. awamori*.

For the other *Agrobacterium* strain, A348, statistical analysis showed that optimal T-DNA transfer also required co-cultivation of at least 2 or 3 days (Table 2B). Furthermore, it seems that co-cultivation of 22.5 or 25 °C is preferred to co-cultivation at 20 °C as, although not always significant, a lower and more variable transformation frequency was obtained. Again, co-cultivation at 28 °C resulted in high background growth of *A. awamori* and decreased transformation frequencies. It should be noted that in the absence of acetosyringone no transfer was seen, indicating that transfer was mediated by the virulence system. In conclusion, by defining the co-cultivation temperature and period, we have determined the optimal and most reproducible T-DNA transfer conditions for LBA1100 (2 or 3 days at 22.5 or 25 °C) and A348 (3 days at 22.5 °C or 2 days at 25 °C).

3.2. Role of virulence proteins in T-DNA transfer to A. awamori

Transformation of filamentous fungi with the *Agrobacterium* system requires induction of the virulence genes with acetosyringone in order to obtain transformants (See above; Covert et al., 2001; de Groot et al., 1998; Malonek and Meinhardt, 2001), indicating that T-DNA transfer to fungi depends on the *vir*-system, like in yeast and plants (Bundock et al., 1995; Zupan et al., 1998). To elucidate the role of the different virulence proteins in T-DNA transfer to *A. awamori*, a set of *Agrobacterium* mutants each deficient in one of its virulence proteins was used to transform *A. awamori* to hygromycin resistance using pUR5750 as a binary

Table 3
Number of transformants after co-cultivation with *Agrobacterium* mutants

Strain	*vir* mutation	Proposed function	Number of transformants/10^6 spores
LBA1100[a]	None		200 ± 25[c]
LBA1141	*virH*	Putative cytochrome P450	200 ± 25
LBA1142	*virA*	Phenolic sensor protein	0
LBA1143	*virB4*	ATPase, transport activation	0
LBA1144	*virB7*	Stabilizer transport pore	0
LBA1145	*virG*	Phenolic response regulator	0
LBA1146	*virC2*	Unknown	15 ± 5
LBA1147	*3′virD2*	T-DNA processing (nuclear targeting signal deleted)	5 ± 5
LBA1148	*virD4*	Coupling factor	0
LBA1149	*virE2*	Protection T-strand against nucleases, facilitating T-strand import into the nucleus	125 ± 15
LBA1150	*virD1*	T-DNA processing	0
LBA1151	*5′virD2*	T-DNA processing (no exonuclease activity)	0
LBA2561	*virF*	Regulator plant cell division cycle	200 ± 25
LBA2565	*virE3*	Unknown	200 ± 25
A348[b]	None		150 ± 25[c]
A348	*virB1*	Transglycosylase (minor component of the T-pilus)	7 ± 3
A348	*virB2–virB11*	Formation of transport pore	0

[a] Co-cultivation was performed for 3 days at 22.5 °C.
[b] Co-cultivation was performed for 2 days at 25 °C.
[c] Values represent the mean of three independent experiments.

vector. Transformation frequencies of these strains were compared to the transformation frequency of the parental *Agrobacterium* strain (Table 3). The following T-DNA transfer conditions 3 days at 22.5 °C and 2 days at 25 °C were used in these experiments for LBA1100 and A348, respectively.

Inactivation of one of the two virulence proteins (VirA, VirG), belonging to the two component regulatory system, resulted in a complete loss of transformants, corroborating that the virulence system needs to be activated to permit T-DNA transfer. Inactivation of VirH, a putative cytochrome P450, had no significant influence on the transformation frequency. Inactivation of the virulence proteins involved in T-DNA processing, like the VirD1 and VirD2 border repeat endonuclease, resulted in a complete loss of transformants. In the absence of VirC2 the transformation frequency was reduced to 8% compared to the wild type. Inactivation of the coupling protein VirD4 and polar mutations in the *virB* operon (VirB4, VirB11) resulted in a complete absence of hygromycin resistant colonies on the selection plates. Inactivation of each of the 11 VirB proteins (non-polar mutations) abolished the formation of transformants, except with VirB1 of which a reduction of 95% in the number of *A. awamori* transformants was observed (Table 3). Similar results were previously observed in plants (Berger and Christie, 1994).

Certain virulence proteins, like VirE2, VirE3, and VirF, are exported independently of the T-DNA to the host during the transformation process (Regensburg-Tuink and Hooykaas, 1993; Schrammeijer et al., 2003; Vergunst et al., 2000). Inactivation of virulence protein E2, a single stranded DNA binding protein involved in protection of the T-strand and in transport of the T-strand into the nucleus, led to a reduction of 37% in the number of *A. awamori* transformants. Inactivation of the virulence proteins VirE3 and VirF, which are host range factors necessary for the transformation of some plant species only (Melchers et al., 1990; Schrammeijer, 2001), had no significant influence on the fungal transformation frequency.

Once the T-strand arrives in the host cytoplasm, it needs to be directed to the nucleus. Two virulence proteins are involved in this process: VirE2 and VirD2. As described above the absence of VirE2 led to a 37% reduction in the number of transformants. A severe reduction of the transformation efficiency was observed when the C-terminally localized nuclear localization signal (NLS) of VirD2 was deleted. Only a few colonies were repeatedly formed (a 97% reduction) on the selection medium indicating an important role of this nuclear localization signal in T-DNA transformation of fungi. The results obtained with the *Agrobacterium* virulence mutants indicate that *Agrobacterium*-mediated transformation of *A. awamori* requires an intact T-DNA transfer machinery for efficient transformation.

4. Discussion

Agrobacterium-mediated transformation is an attractive method for the transformation of filamentous fungi. Based on the requirement of acetosyringone in order to obtain transformants and on the T-DNA structure of the integrated T-DNA it was inferred that the transformation was mediated by the virulence sys-

tem of *A. tumefaciens*. Although, this was shown for the yeast *S. cerevisiae* (Bundock et al., 1995), this was so far not shown directly for filamentous fungi. To fill this gap we have now studied fungal transformation by different *Agrobacterium* mutants deficient in one of the virulence proteins to determine the role of that particular virulence protein in T-DNA transfer to the filamentous fungus *A. awamori*.

As the results of initial experiments, carried out at 'room temperature,' were somewhat variable, we first optimized the system for *Agrobacterium*-mediated transformation of *A. awamori* for two *Agrobacterium* strains in order to obtain reproducible high transformation efficiency. By optimization of co-cultivation time and temperature a higher transformation frequency than the reported frequency by de Groot et al. (1998) was reproducibly obtained. We found that both the length and temperature of the co-cultivation period are important variables to take into consideration. Incubation at 22.5 and 25 °C for a period of two or three days was sufficient to obtain maximal transformation. Prolongation of the co-cultivation period to three days 28 °C resulted in a background growth of *A. awamori* and in a reduction in the transformation frequency. However, a variation in the transformation frequency between the three independent experiments still existed and were more pronounced at 20 and 28 °C. It is known that both 20 and 28 °C are not the optimal temperatures for T-DNA transfer (Dillen et al., 2002; Salas et al., 2002) and it could be that these sub-optimal conditions for T-DNA transfer are more sensitive to small variations in the temperature or (other) unknown factors. Southern blot analysis of transformants obtained from the co-cultivation conditions 3 days at 22.5 °C or 2 days at 25 °C showed that in both cases around 30% of the transformants contained multiple T-DNA insertions (C.B. Michielse, unpublished). Our results indicate that there is an optimal balance between co-cultivation temperature and period in order to obtain a maximum number of transformants in *Agrobacterium*-mediated transformation. Also in plants it has been shown that optimal co-cultivation condition and hence a high transformation frequency is dependent on the combination of an efficient *Agrobacterium* T-DNA transfer machinery assembly and on the susceptibility of the host organism for stable T-DNA integration (Dillen et al., 2002; Fullner and Nester, 1996; Salas et al., 2002).

T-DNA transfer to *A. awamori* was shown to be dependent on an intact virulence system of *A. tumefaciens*. Inactivation of VirA, VirG (two component regulatory system), VirD1, VirD2 (deficient in T-strand generation), and coupling factor VirD4 completely abolished the formation of transformants. Deletion of the C-terminally located NLS and omega sequence of VirD2 resulted in a very low transformation frequency by *Agrobacterium*.

Initially, this mutant did not give any T-DNA transfer to *S. cerevisiae* (Bundock et al., 1995). However, after optimization of the *Agrobacterium*-mediated transformation of *S. cerevisiae* we found a very low transformation frequency of 0.2% compared to the wild type *Agrobacterium* strain (A. den Dulk-Ras, C.B. Michielse, P.J.J. Hooykaas, unpublished). It can be concluded that the NLS and omega sequence are important, but not absolutely essential for T-DNA transfer to *S. cerevisiae* and *A. awamori*. In plants, the situation is similar as mutations in the C-terminus of VirD2 severely attenuate or abolish tumorigenesis (Bravo-Angel et al., 1998; Koukolikova-Nicola et al., 1993; Shurvinton et al., 1992). It is remarkable that after deletion of the NLS sequence of VirD2, still some T-DNA complexes manage to be translocated into the nucleus, giving rise to a small number of transformed fungal or plant cells. One explanation for this residual T-DNA transfer to the host nucleus may be due to the action of VirE2, which might target the T-DNA to the nucleus. However, a double *virE2*/*virD2*ΔNLS mutant assayed on *Nicotiana tabacum* still showed residual T-DNA transfer (Rossi et al., 1996). Therefore, it seems likely that the residual T-DNA transfer to *A. awamori* observed in transformations with the *3′virD2 Agrobacterium* mutant is not due to the presence of the VirE2 protein. The residual transfer could be due to the fact that the T-DNA might reach the nucleus by chance or via an alternative pathway. To investigate the exact function of VirE2 in the residual T-DNA transfer in *A. awamori*, the transformation efficiency of a double *virE2*/*virD2*ΔNLS mutant should be determined.

Disruption of one of the VirB proteins, except VirB1 also reduced the transfer efficiency to zero. An intact VirB machinery, except VirB1, is required for T-pilus formation and hence for T-DNA transfer (Schmidt-Eisenlohr et al., 1999). Inactivation of VirB1 reduced the T-DNA transfer efficiency 10- to 100-fold in *Nicotiana* and *Kalanchoë* (Berger and Christie, 1994; Llosa et al., 2000). Virulence protein B1 is a putative lytic transglycosylase and might provide local lysis of the peptidoglycan cell wall necessary for transport complex assembly (Lai and Kado, 1998). Therefore, inactivation of VirB1 might lead to reduced VirB transporter assembly and consequently a reduced T-DNA transfer as observed in the *A. awamori* transformations. This indicates that translocation of the T-DNA to the transport pore and pore assembly plays an essential role during *Agrobacterium*-mediated transformation of filamentous fungi.

Proteins encoded by the *virA*, *virG*, *virD*, and *virB* loci have been shown to be important or necessary for efficient T-DNA transfer to plants and yeast (Bundock et al., 1995; Gelvin, 2000). However, proteins of *virC*, *virE*, *virF*, and *virH* loci are only necessary for T-DNA transfer to certain host species (Gelvin, 2000;

Zambrysky, 1992). The virulence proteins encoded by the *virC* locus are not absolutely required for pathogenicity, but they might contribute to T-DNA transfer efficiency and thereby to the degree of virulence (Yanofsky and Nester, 1986). *VirC1* and *virC2* mutants show severely attenuated virulence on *Kalanchoë* species, but less effect is seen on sunflower and tomato (Close et al., 1987). In vivo nicking of T-DNA decreases in both *virC1* and *virC2* mutants, thereby suggesting that proteins encoded by the *virC* locus might play a role in enhancement of nicking (Toro et al., 1988). In agreement with this suggestion it was shown that VirC1 binds to the 'overdrive' sequence located at the right T-DNA border and thereby increasing T-DNA processing. Furthermore, it was shown that VirC2 was not required for interaction of VirC1 with the 'overdrive' sequence (Toro et al., 1988, 1989). Inactivation of VirC2 led to a reduction in the number of transformants in *Agrobacterium*-mediated transformation of *A. awamori* and *S. cerevisiae* (A. den Dulk-Ras, C.B. Michielse, P.J.J. Hooykaas, unpublished), indicating that VirC2 is necessary for a high level of T-DNA transfer. A reduction in transient T-DNA transfer of this particular *virC2* mutant was also observed for *Nicotiana glauca* (Mozo and Hooykaas, 1992).

Many functions have been described for virulence protein E2, like protection of the T-strand against nucleases, facilitating T-strand transport through the nuclear pore and keeping the T-strand in an unfolded state (Ward and Zambryski, 2001). Inactivation of the VirE2 protein only reduced the transformation frequency of *A. awamori* to 63%. This is in contrast to what has been observed in plants and yeast. *Agrobacterium virE2* mutants assayed on plants are highly attenuated in virulence, although on some plant species, these mutants are capable of inciting tumours at a very low efficiency (Dombek and Ream, 1997; Stachel and Nester, 1986). Transient T-DNA transfer of an *Agrobacterium virE2* mutant assayed on tobacco was reduced to 0.03% compared to wild type (Rossi et al., 1996). In yeast the level of T-DNA transfer is intermediate between that observed in plants and *A. awamori*. Bundock et al. (1995) found that an *Agrobacterium virE2* mutant reduced the level of T-DNA transfer to *S. cerevisiae* to 10% compared to wild type. These results indicate that there is a different requirement for VirE2 between the hosts. This could be, for instance, due to differences in the amount of nucleases capable of degrading the T-strand or due to differences in efficient nuclear targeting of the T-strand in the absence of VirE2 between the hosts. Nevertheless, it can be concluded that VirE2 does not play an essential role in transformation of *A. awamori*, but it is necessary for optimal T-DNA transfer.

VirF and VirH do not seem to play an active role in the transformation of *A. awamori*. Inactivation of these proteins, known as host range factors, had no effect on the transformation frequency. Also absence of the VirE3 protein did not have an effect on the transformation frequency of *A. awamori*. The biological function of VirE3 is not known. Schrammeijer (2001) described a mild or no effect of VirE3 deletion in tumour assays. However, a double deletion mutant *virF* and *virE3* led to a strong diminished tumour formation on some plants, but not on others. Therefore, to fully study the role of VirF and VirE3 in *Agrobacterium*-mediated transformation of *A. awamori* a double deletion mutant should be used to determine the role of these two virulence proteins in T-DNA transfer.

Finally, it can be concluded, based on the observations made in this study, that *Agrobacterium*-mediated transformation of *A. awamori* and probably also other filamentous fungi is dependent on an intact virulence system of *A. tumefaciens*. Furthermore, it can be concluded that the virulence proteins VirE3, VirH, and VirF are not required for optimal T-DNA transfer, whereas, VirC2, VirE2, and the C-terminal part of VirD2 are required for efficient T-DNA transfer.

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