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Development of a system for integrative and stable transformation of the zygomycete *Rhizopus oryzae* by *Agrobacterium*-mediated DNA transfer

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Abstract Two transformation systems, based on the use of $CaCl_2$/PEG and *Agrobacterium tumefaciens*, respectively, were developed for the zygomycete *Rhizopus oryzae*. Irrespective of the selection marker used, a *pyr4* marker derived from *R. niveus* or a dominant *amdS*$^+$ marker from *Aspergillus nidulans*, and irrespective of the configuration of the transforming DNA (linear or circular), the transformants obtained with the $CaCl_2$/PEG transformation method were found to carry multiple copies of tandemly linked vector molecules, which failed to integrate into the genomic DNA. Furthermore, these transformants displayed low mitotic stability. In contrast, transformants obtained by *Agrobacterium*-mediated transformation were mitotically stable, even under non-selective conditions. Detailed analysis of these transformants revealed that the transforming DNA had integrated into the genome of *R. oryzae* at a single locus in independently obtained transformants. In addition, truncation of the transforming DNA was observed, resulting in the integration of the *R. niveus pyr4* marker gene, but not the second gene located on the transferred DNA. Modification of the transforming DNA, resulting in partial resistance to restriction enzyme digestion, was observed in transformants obtained with the $CaCl_2$/PEG transformation method, suggesting that a specific genome defence mechanism may exist in *R. oryzae*. It is likely that the unique mechanism used by *A. tumefaciens* to deliver its transferred DNA to its hosts facilitates bypass of the host defence mechanisms, thus allowing the DNA to integrate into the chromosomal genome.

Keywords Zygomycete · *Rhizopus oryzae* · Transformation · *Agrobacterium tumefaciens*

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Introduction

Rhizopus oryzae, a filamentous fungus belonging to the class of zygomycetes, is an important industrial microorganism used for the commercial production of organic acids (Misro et al. 1997; Skory 2002) and industrial enzymes (Thompson and Eribo 1984; Amadioha 1993; Tunga et al. 1999).

Recently, genes involved in the degradation of cellulose (Moriya et al. 2003) and the conversion of pyruvate to ethanol (Skory 2002, 2003) have been cloned from *R. oryzae*. However, to permit functional analysis of cloned genes and allow genetic modification of this organism, an integrative transformation system is required (1) to ensure the stability of the genetic information introduced, and (2) to develop systems that make gene targeting and gene replacement possible.

Transformation systems have been described for a small number of zygomycetes, including *Absidia glauca* (Wöstemeyer et al. 1987; Burmester et al. 1990), *Mucor circinelloides* (van Heeswijck et al. 1988; Anaya and Roncero 1991; Benito et al. 1992; Iturriaga et al. 1992; Ruiz-Hidalgo et al. 1999), *Parasitella simplex* (Burmester 1992), *Phycomyces blakesleeanus* (Revuelta and Jayaram 1986; Arnau et al. 1988; Suarez and Eslava 1988) and *Rhizomucor pussilis* (Wada et al. 1996; Yamazaki et al. 1999). Although targeted integration upon the application of strong selection for marker stability has been reported for *R. pussilis* (Wada et al. 1996; Yamazaki et al. 1999) and *M. circinelloides* (Arnau et al. 1991; Arnau and Stroman 1993), it is not the normal fate of the introduced DNA. In most of the transformation systems so far described for zygomycetes, the transforming DNA remains extrachromosomal, replicating autonomously and giving rise to transformants which display low mitotic stability (Revuelta and Jayaram 1986; Wöstemeyer et al. 1987; Anaya and Roncero 1991; Burmester 1992; Iturriaga et al. 1992; Benito et al. 1995).

Versatile genetic modification of zygomycetes is thus severely hampered by the lack of an integrative transformation system, which would be expected to enhance the mitotic stability of transformants. Several attempts have been made to establish a stable and/or integrative transformation system for zygomycetes. Thus, Burmester et al. (1992) reported that the mitotic stability of *A. glauca* transformants could be increased by the inclusion of a SEG1 element on the introduced DNA. This resulted in stabilization of the autonomously replicated DNA in *A. glauca*, even under non-selective conditions; however, chromosomal integration was not observed (Burmester et al. 1992). Integration into the genome of *A. glauca* was obtained by the incorporation of repetitive DNA elements into an autonomously replicating plasmid. However, these integration events were often associated with rearrangements of the introduced DNA and with the appearance of mutant phenotypes (Burmester et al. 1990). In the zygomycete *Mortierella alpina* a homologous ribosomal DNA region was included in the vector used to obtain stable transformants. However, 25–30% of the transformants remained unstable due to failure of the plasmid to integrate or to rearrangements of integrated plasmids (Mackenzie et al. 2000).

Transformation systems have also been developed for species that are closely related to *R. oryzae*, such as *R. niveus* and *R. delemar*, but these systems too result in autonomous replication of the introduced DNA and in low mitotic stability of transformants (Yanai et al. 1990, 1991; Liou et al. 1992; Horiuchi et al. 1995; Takaya et al. 1996). Possible integration of the introduced DNA in multiple tandem arrays has been reported by several groups (Liou et al. 1992; Horiuchi et al. 1995; Takaya et al. 1996). However, in these cases, integration was inferred from hybridization data for undigested chromosomal DNA. Introduced DNA which replicates in a concatenated high-molecular-weight form can co-migrate with genomic DNA (Skory 2002; this study), and therefore, the status of the transforming DNA in *R. niveus* and *R. delemar* in these systems remains unclear. More recently, a transformation system for *R. oryzae* based on a biolistic method has been described (Skory 2002). Introduction of the DNA in a circular form led predominantly to autonomous replication and yielded mitotically unstable transformants. Introduction of a linear DNA fragment was the most effective method for chromosomal integration, although autonomous replication of the introduced DNA still occurred in 40% of the transformants (Skory 2002). Thus, although integration of the introduced DNA into the genome of zygomycetes can occur, it is rare and is often correlated with rearrangements of the transforming DNA.

In order to develop a stable and integrative transformation system for *R. oryzae*, we have compared an auxotrophic (*pyr4*) selection marker with a dominant selection marker (*amdS*), circular with linear transforming DNA, and the $CaCl_2$/PEG method with *Agrobacterium*-mediated transformation. From these experiments we concluded that only the combination of *Agrobacterium*-mediated transformation with the *pyr4* selection marker resulted in integrative transformation of *R. oryzae*, yielding transformants that showed 100% mitotic stability.

Materials and Methods

Strains, plasmids and culture conditions

R. oryzae COM1291, a *pyr4* $^{-}$ derivative of COM1247 (Goldberg et al. 1991), was generated by nitrosoguanidine mutagenesis and used as the recipient strain for fungal transformation. COM1291 was cultured on agar plates containing V8 medium [containing 50% V8 vegetable juice (Campell Grocery Products Ltd.), 2% glycerol, 3% agar and 10 mM uridine]. *Escherichia coli* DH5α (GibcoBRL) and JM109 (Stratagene) served as hosts for the construction, propagation and amplification of the plasmids used in this study. Recombinant plasmids were constructed using the vectors pMTL22, pMTL23 (Chambers et al. 1988), pUC19 (Vieira and Messing 1982), pBIN19 (Bevan 1984), pLPGUS21 (Takaya et al. 1994) and pRNU54 (Horiuchi et al. 1995). The primers used in this study are listed in Table 1. *E. coli* was grown in LB medium (Sambrook et al. 1989) containing either ampicillin (50 μg/ml) or kanamycin (25 μg/ml), depending on the resistance marker on the plasmid used. The *A. tumefaciens* strain LBA1100 (pAL1100ΔT-DNA, Δ*tra*, Δ*occ*; Beijersbergen et al. 1992), containing pKS118 (this study), was used for *Agrobacterium*-mediated transformation, and was grown in LB medium containing spectinomycin (250 μg/ml) and kanamycin (100 μg/ml) at 28°C. Introduction of pKS118 into LBA1100 was performed as described by Mattanovich et al. (1989).

Table 1 Primers used in this study

Primer	Sequence (5' to 3')[a]
PSTSALXBA	CTGCAGGTCGACTCTAGA
PGK2ATG	GAAGATCTAGAAGACGGCATGC TGTTGATTG
p *amdS*ATG	CGGAATTCGTCTCGCATGCCTCAAT CCTGG
p *amdS*TAG	CGGAATTCCTATGGAGTCACCACATT TCCC
p *amdS*550	CAACAACATCATCCGG
p *amdS*600	ACCAGAACTGCCGCC
p *amdS*1250	ACTTCTGGAGATGCG
*pyr4*ATG	ACATGCATGCTCATGAACACATACA AGCC
*pyr4*STOP	GCTCTAGACTATTTATTGTGTAAAGC
KS-*nptII*-1	GTGGGCGAAGAACTCCAGCA
KS-*nptII*-2	CTTGACGAGTTCTTCTGAGC
KS-*nptIII*-sens	GGAGCACCTCAAAAACACCATCATA
KS-*nptIII*-reverse	GAGAGCCAAAACACTTGATGCG
pBINp2	CGACCTGTACCATTCGGTGAG
pBINp3	CGGAAGAACGGCAACTAAGC
pBINp4	CTGATGGGCTGCCTGTATCG
pBINp5	TGCTGCAACTCTCTCAGGG
pBINp6	GATAGGGTTGAGTGTTGTTCC
pBINp7	CAACTTAATCGCCTTGCAGC
pBINp8	TGTTATCCGCTCACAATTCC
pBINp9	TGCTATCGATGGTTTCATTGG
pyr 2	GAGCATATGTGATGGATTGAG
pyr 4	GGTTTCACGAATTCAAGAGTG
pyr 7	GCACAGAGAACGATTTGATCA
pyr 8	CAGCTATTGACGCGTGATCC
pyr 9	TTGGTCCTGGCTTGTCTACC
pyr 10	GCAACATGTTCAATCATCCAG
pyr 11	TTCCGATCAATATTTTACTGACC
nos 1	GAATCCTGTTGCCGGTCTTG
nos 2	TTATCCTAGTTTGCGCG

[a]Restriction enzyme recognition sites are *underlined*

Construction of recombinant plasmids

The expression vector pRN1A carries the *R. niveus pgk2* promoter, the *amdS* gene from *Aspergillus nidulans* as a selection marker, and the *R. niveus pyr4* terminator. This plasmid was constructed as follows. The promoter region was isolated by PCR using as primers PSTSALXBA and PGK2ATG (which contain convenient cloning sites overlapping the ATG start codon) and pLPGUS21 as the template. The *Bgl*II + *Xba*I-digested PCR product was cloned into pMTL23, resulting in pMTLPGK. A *Bcl*I-*Nae*I fragment containing the *pyr4* termination region was isolated from pRNU54 and cloned into pMTLPGK digested with *Bgl*II and *Stu*I, resulting in pPGKEXV. A basic expression vector (pRN1) consisting of the *pgk2* promoter and the *pyr4* terminator was constructed by introducing a *Bam*HI-*Xba*I fragment of pPGKEXV into pMTL22 digested with *Bam*HI and *Nhe*I. The *amdS* selection marker, devoid of introns, was amplified by RT-PCR from a multicopy *A. niger amdS* transformant (Hanegraaf et al. 1991). RT-PCR amplification was carried out using M-MLV-RT (GibcoBRL) for reverse transcription, followed by PCR with the primers p *amdS*ATG and p *amdS*TAG using LongExpand polymerase (Roche) for 10 cycles of 1 min at 94°C, 1 min at 42°C and 2 min at 68°C, followed by 10 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 68°C. The cDNA was cloned as an *Eco*RI fragment into pUC19 and its sequence was verified by performing a sequence reaction on a Perkin Elmer ABI PRISM 310 using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and the primers p *amdS*ATG, p *amdS*TAG, p *amdS*550, p *amdS*600 and p *amdS*1250 (Table 1). An *Sph*I-*Xba*I fragment of pUC19-*amdS* was cloned into pRN1 digested with *Sph*I and *Xba*I, resulting in vector pRN1A. The vector pRN1A *pyr4*, which carries both selection markers, was constructed by inserting the *Bgl*I-*Pvu*II fragment of pRN1A (containing *amdS*) into pRNU54 digested with *Bgl*I and *Nae*I.

The binary vector used for *Agrobacterium*-mediated transformation was made by digesting pRNU54 with *Xba*I and religating, thereby deleting 730 bp downstream of the *pyr4* terminator region. The resulting plasmid was named pKS109. The *pyr4*-containing *Bam*HI fragment of pKS109 was cloned into pBIN19 digested with *Bam*HI, resulting in pKS118.

Preparation of protoplasts

The *R. oryzae* strain COM1291 was grown for 3 days at 34°C on agar plates containing V8 medium. Sporangiospores were isolated with a physiological salt solution and subsequently inoculated, to a final concentration of 10^7 spores/ml, into 25 ml of YPG (Yanai et al. 1990) supplemented with 10 mM proline and 10 mM uridine. Germlings were obtained after incubation on a rotary shaker (at 350 rpm) of 6–7 h at 34°C. Germlings were collected by centrifugation for 10 min at 900× *g*, washed once with physiological salt, and then resuspended in 25 ml of salt buffer (0.27 M $CaCl_2$, 0.6 M NaCl) containing 50 mg/ml Glucanex (Novozyme) and 5 mg/ml Caylase M3 (Cayla). After incubation for 15–16 h on a rotary shaker (100 rpm) at 30°C, the protoplasts were collected by filtration through Miracloth, suspended in 25 ml of STC (1.2 M sorbitol, 10 mM TRIS-HCl pH 7.5, 50 mM $CaCl_2$, 35 mM NaCl) and incubated on ice for 5 min, pelleted by centrifugation for 10 min at 900× *g*, washed once with STC and resuspended in 1 ml of fresh STC. Aliquots of these protoplasts were used both for $CaCl_2$/PEG- and *Agrobacterium*-mediated transformation.

$CaCl_2$/PEG transformation

An aliquot of the protoplast suspension (100 µl) was mixed with 10–20 µg of DNA and 1 µl of 1 M aurin tricarboxylic acid. After incubation on ice for 5 min, 10 µl of PEG solution [40% (w/v) PEG4000, 10 mM MOPS (pH 6.3), 50 mM $CaCl_2$] was added, and the mixture was incubated on ice for a further 25 min. The DNA-protoplast mixture was gently mixed with 1.25 ml

of PEG solution, and incubated at room temperature for 25 min; then 7 ml of STC was added and the protoplasts were collected by centrifugation for 10 min at 900× *g*. The pellet was resuspended in 300 µl of STC, which was then spread on agar plates containing RMM medium (0.6 M sorbitol, 0.2% asparagine, 2% glucose, 1 mM $MgSO_4$, 0.5 g/l KH_2PO_4, 0.1% casamino acids and 1.5% agar) for *pyr4* selection, or onto agar plates containing acetamide medium (0.6 M sorbitol, 2% glucose, 0.5 g/l KH_2PO_4, 1 mM $MgSO_4$, 10 mM acetamide, 2 mM uridine and 1.5% agar) for *amdS* selection, and incubated for 3–4 days at 34°C.

Agrobacterium-mediated transformation

An aliquot of the protoplast mixture (100 µl) was mixed with induced *Agrobacterium* cells obtained as previously described (Michielse et al. 2004b). The mixture was spread onto Hybond-N filters (Amersham), which were placed on agar plates containing induction medium (Bundock et al. 1995). Co-cultivation was performed for 1 day at 28°C, and the filters were subsequently transferred to agar plates containing SIV medium pH 3 (2% glucose, 0.2% urea, 5 mg/ml KH_2PO_4, 0.5 mg/ml $MgSO_4{\cdot}7H_2O$, 0.28 mg/ml $CaCl_2{\cdot}2H_2O$, 20 µg/ml citric acid, 15 µg/ml $Fe(NO_3)_3{\cdot}9H_2O$, 10 µg/ml $ZnSO_4{\cdot}7H_2O$, 1.8 µg/ml $MnSO_4{\cdot}H_2O$, 0.5 µg/ml $CuSO_4{\cdot}5H_2O$, 0.5 µg/ml $NaMoO_4{\cdot}2H_2O$, 1.5% agar) and incubated for 3–4 days at 34°C.

Assay for mitotic stability of transformants

The mitotic stability of the *R. oryzae* transformants was determined as follows. After two purification rounds on agar plates containing RMM plus 0.1% Triton, spores from a single colony were used to inoculate a spore plate (containing RMM). After incubation for 3 days at 34°C, spores were harvested, diluted to 10^3 spores/ml and spread onto agar plates containing RMM and RMM supplemented with 10 mM uridine. Mitotic stability was expressed as the ratio of $pyr4^+$ colonies on RMM plates to the total number of colonies that grew on RMM + uridine plates.

Isolation and Southern analysis of fungal chromosomal DNA

R. oryzae transformants were grown overnight in 50 ml of RMM medium (10^6 spores/ml) at 34°C, and total chromosomal DNA was isolated as described by Kolar et al. (1988). For Southern analysis aliquots (0.5–1 µg) of chromosomal DNA were digested with 10 U of restriction enzyme as specified in the Results section, and incubated overnight at 37°C. The samples were then loaded on a 1% 0.5×TRIS-borate/EDTA gel and electrophoresed for 18 h at 40 V. Digested DNA was transferred to Hybond-N^+ (Amersham Pharmacia) either by vacuum blotting as described by the manufacturer (VacuGene XL, Amersham Pharmacia) or by capillary transfer (Sambrook et al. 1989). The probes used for Southern analysis of transformants obtained by the $CaCl_2$/PEG method were pRNU54 and pRN1A *pyr4*. For analysis of the transformants obtained by *Agrobacterium*-mediated transformation probes were prepared by PCR, using the primers *pyr4*ATG and *pyr4*STOP to amplify the *pyr4* ORF. For detection of the *nptII* and *nptIII* genes located on the T-DNA and the binary vector, an *nptII* probe was obtained using the primers KS-*nptII*-1 and KS-*nptII*-2, while *nptIII* was amplified with the primers KS-*nptIII*-sens and KS-*nptIII*-reverse. To localize T-DNA breakpoints ten PCR products spanning the T-DNA region were generated using the following primer pairs: pBINp2-pBINp3, pBINp4-pBINp5, pBINp6-pBINp7, *pyr* 8-*pyr* 9, *pyr* 4-*pyr4*ATG, *pyr* 2-*pyr* 10, *pyr* 11-*pyr* 7, pBINp8-pBINp9, *nos* 1-*nos* 2 and KS-*nptII*-1-KS-*nptII*-2. The resulting amplification products correspond to the pBIN19 backbone vector sequence, the left T-DNA border, pBIN19 T-DNA (M13 ori), the *pyr4* terminator, the *pyr4* ORF, the *pyr4* promoter (−160 to 840 bp), the *pyr4* promoter (−990 to 1159 bp), pBIN19 T-DNA (gene III), the *nos* terminator, and *nptII* (see below), respectively. The Rediprime II DNA Labelling System (Amersham Pharmacia) was used to label the probes with [α^{32}P]dCTP. Hybridization was carried out as described in the protocol supplied with GeneScreen (DuPont NEN Research Products). Hybridization signals were detected witth a Phosphor Imager (Molecular Dynamics) or by autoradiography (using Kodak X-ray film and Kodak intensifying screens at –80°C).

Results

$CaCl_2$/PEG-mediated transformation with circular and linear DNA molecules

An auxotrophic *pyr4* mutant of *R. oryzae*, generated by nitrosoguanidine mutagenesis, was transformed to prototrophy using $CaCl_2$/PEG-mediated transformation and a circular pRNU54 plasmid containing the *pyr4* gene of *R. niveus*. Molecular analysis of the *pyr4* mutant revealed that the mutant had a 54-bp deletion in the *pyr4* allele, allowing discrimination of the wild-type and mutant alleles by Southern analysis. In total, 13 transformants were obtained, corresponding to a transformation frequency of 0.1 transformants/µg of DNA. Analysis of the transformants demonstrated that their mitotic stability was low, and ranged from 0.01 to 5%. Repeated rounds of single-colony purification have been reported to increase the mitotic stability of transformants (Horiuchi et al. 1995). However, in our hands, the mitotic stability of the transformants did not increase despite several rounds of purification (data not shown). Southern analysis of the transformants revealed

fragments corresponding to intact pRNU54. Figure 1A shows fragments of 5.5, 1.0 and 0.8 kb, and additional fragments suggested to be due to incomplete digestion caused by modification of restriction sites. These data indicate that the plasmid had not integrated into the genomic DNA, but replicated autonomously. CHEF analysis was performed on unstable transformants, and confirmed that they carried multiple copies of tandemly linked vector molecules, which were not integrated into the genome (data not shown).

As their low mitotic stability indicated, and the Southern analysis confirmed, free plasmids were present in the transformants. We therefore attempted to rescue these plasmids into *E. coli*. Undigested chromosomal DNA from five characterized transformants was used for electroporation of *E. coli* JM109. A small number of ampicillin-resistant *E. coli* colonies was obtained, thereby confirming that autonomously replicating, free plasmids existed in the transformants. No transformants were obtained after electroporation of *E. coli* with untransformed chromosomal DNA from the parental *R. oryzae* strain, COM1247. Restriction analysis of the plasmids in these *E. coli* transformants revealed in most cases the presence of unmodified pRNU54. However, in 21% of the re-isolated plasmids a modified restriction pattern was observed due to deletions of short segments of the vector sequence (data not shown).

In an attempt to improve the rate of integration of vector DNA into the fungal genome, a linear DNA fragment carrying the *pyr4* selection marker (on the 5.6-kb *Sph*I fragment of pRNU54) was transformed into *R. oryzae*. In a parallel experiment the 5.6-kb fragment was pre-treated with DNA-ligase and introduced as a circular molecule. The transformation frequency obtained was comparable to that observed with pRNU54 (0.15 transformants/μg DNA for the linear 5.6-kb fragment and 0.1 transformants/μg DNA for the ligated 5.6-kb fragment). The mitotic stability of the transformants obtained with the linear and ligated 5.6-kb fragments was low and varied from 0 to 5%, indicating that in both cases the introduced DNA was replicating autonomously. Autonomous replication due to re-ligation of linear fragments introduced into *R. oryzae* has also been observed by Skory (2002). Autonomous replication of the DNA introduced into the *R. oryzae* transformants generated in this study was confirmed by Southern analysis. Fragments corresponding to the ligated 5.6-kb fragment were observed in transformants obtained with the pre-ligated 5.6-kb fragment (Fig. 1B; 4.2, 1.0, 0.8, 0.4 and 0.3 kb). Southern analysis of the transformants obtained with the linear 5.6-kb fragment revealed three of the five expected fragments (0.8, 0.4 and 0.3 kb), but not the 3.1- or 1.0-kb fragment. Instead two additional fragments (6.2 and 2.0 kb) were observed, which result from the re-ligation of multiple linear molecules (Fig. 1B). It should be noted that, in all cases, the Southern analysis revealed additional fragments (Fig. 1A and B). Due to the low mitotic stability of the transformants, it is unlikely that these fragments

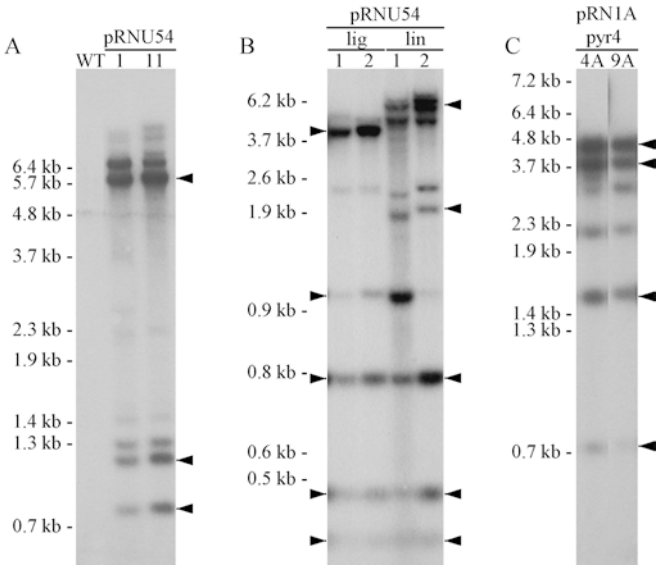

Fig. 1

result from integration of the transforming molecules into the genome. Instead, we suggest that the additional fragments are the result of modification of some of the *Eco*RI and *Bam*HI restriction sites present in the introduced molecules, resulting in incomplete digestion by these enzymes.

$CaCl_2$/PEG-mediated transformation using the dominant selection marker *amdS*

As an alternative to the non-integrating *pyr4* marker, a dominant selection marker, *amdS*, which had not been previously used for zygomycete transformation was tried. The vector pRN1A *pyr4*, carrying *amdS* and *pyr4* as selection markers, was used to compare the efficacy of the two selection markers for *R. oryzae* transformation. The double selection vector, pRN1A *pyr4*, was introduced into *R. oryzae*, and primary transformants were first selected for *pyr4* expression and subsequently tested for *amdS* expression. A total of 24 transformants was obtained (0.08 transformants/μg DNA) based on *pyr4* selection. The mitotic stability of 23 out of the 24 transformants analyzed ranged from 0 to 3.5% for the *pyr4* marker, again indicating that the plasmid was replicating autonomously. The remaining transformant had a mitotic stability of 48% for the *pyr4* marker. Further analysis revealed that in a *pyr4*$^+$ derivative of this transformant the mutant phenotype had been complemented without leaving vector sequences in the transformed DNA (data not shown). Only five transformants were able to grow on acetamide as sole nitrogen-source. These five *pyr4*/*amdS*-positive transformants were analyzed further for the stability of both markers. Selection of these transformants for the *pyr4* phenotype alone after one purification round revealed low stability of the *pyr4* marker and even lower stability of the *amdS*

Table 2 Mitotic stability of the *pyr4* and *amdS* marker in [pRN1A *pyr4*]-transformants

Transformant	Mitotic stability of *pyr4* (%)	Mitotic stability of *amdS* (%)
[pRN1A *pyr4*]#4[a]	0	0
[pRN1A *pyr4*]#4A[b]	1.3	1.9
[pRN1A *pyr4*]#9	1.5	0
[pRN1A *pyr4*]#9A	4.3	1.3
[pRN1A *pyr4*]#18	3.5	0.003
[pRN1A *pyr4*]#18A	3	0.02
[pRN1A *pyr4*]#22	1.3	0
[pRN1A *pyr4*]#22A	5.5	0.1
[pRN1A *pyr4*]#23	0.6	0
[pRN1A *pyr4*]#23A	4	0.6

[a]Spores isolated from agar plates containing RMM lacking uridine
[b]Spores isolated from agar plates containing acetamide medium without uridine

phenotype (Table 2). Selection for the presence of both markers after one purification round resulted in a slightly higher level of mitotic stability for the *pyr4* and *amdS* markers (Table 2). Southern analysis confirmed the presence of autonomously replicating vector molecules (Fig. 1C; 5.0, 3.8, 1.5, 0.75 kb, and additional fragments suggested to be due to modification of restriction sites).

In order to investigate whether stable integrants could be obtained by mutagenesis, several of the transformants obtained with pRN1A *pyr4* ($pyr4^+/amdS^-$) were mutagenized with UV light or NTG to a survival rate of 1–30%. Mutagenized spores were plated onto agar plates containing acetamide to select for *amdS*-positive clones. Some 5×10^{10} spores were mutagenized; however, no mutants that could grow on acetamide as the sole source of nitrogen were obtained.

Agrobacterium-mediated transformation using the auxotrophic *pyr4* marker

A. tumefaciens has been shown to be able to transfer a part of its DNA (Transferred DNA, or T-DNA) to different fungal species, and this DNA is capable of integrating into the chromosomes (de Groot et al. 1998). Introducing DNA by *Agrobacterium*-mediated transformation might favour DNA integration due to the single stranded nature of the T-DNA and due to the presence of the DNA binding proteins VirD2 and VirE2. To examine whether *A. tumefaciens* could also transfer its T-DNA to *R. oryzae*, a transformation protocol was developed using the *pyr4* selection marker.

Co-cultivation of *A. tumefaciens* with protoplasts of *R. oryzae* resulted in the isolation of numerous *pyr4* prototrophic transformants. Due to the vigorous growth of *R. oryzae* on the transformation plates no single transformants could be counted; therefore, no attempt was made to determine the transformation frequency. Small mycelium plugs from eight different transformation plates were transferred to a new selection plate, and, after two purification rounds, spores from a single colony were used to inoculate a spore plate. Spores were isolated and used for subsequent analysis. Mitotic analysis revealed that in all eight independently obtained transformants the *pyr4* marker displayed high mitotic stability (Table 3). Even after three sporulation rounds under non-selective conditions, the transformants retained the *pyr4* marker (Table 3), indicating that the T-DNA might indeed have integrated into the genome. Southern analysis was performed in order to characterize these transformants further. The analysis revealed that two types of transformants were obtained. In six of the eight transformants an extra copy of the *pyr4* sequence was present (Type I transformants, Fig. 2B). The other two transformants had undergone gene conversion (type II transformants), since no additional copy of *pyr4* could be detected and the endogenous *pyr4* signal in the *Bgl*II digest had shifted from 5.5 to 6 kb (Fig. 2B). Furthermore, PCR analysis using primers spanning the 54-bp deletion present in the *pyr4* gene of the recipient strain revealed that the type II transformants no longer contained this deletion (data not shown). Because only limited information on the sequence of the *pyr4* locus is available, no map of the locus could be constructed for these transformants.

The fate of the T-DNA in the type I transformants was analyzed in further detail. The intensity of the T-DNA signal is comparable to that of the endogenous *pyr4* locus, indicating that one T-DNA copy is present in these transformants (Fig. 2B). After digestion of the chromosomal DNA with various restriction enzymes it was found that single-copy T-DNA integration had occurred at the same locus in all six transformants, suggesting a 'hot spot' for integration. Hybridization of the chromosomal DNA of the transformants with *nptIII* (present on the backbone of the binary vector) and *nptII*, present on the T-DNA (Fig. 2A), did not result in a signal (data not shown), indicating that no vector sequences outside the borders of the T-DNA were present. Furthermore, the results also indicated that the T-DNA itself is incomplete. Truncation of the T-DNA was confirmed by digestion of the chromosomal DNAs from

Table 3 Mitotic stability of the *pyr4* marker in transformants obtained by *Agrobacterium*-mediated transformation

Transformant	Mitotic stability of *pyr4* under selective conditions (%)[a]	Mitotic stability of *pyr4* under non-selective conditions (%)[a]
[pKS118]#1	85	108
[pKS118]#2	98	92
[pKS118]#3	92	105
[pKS118]#4	110	81
[pKS118]#5	102	106
[pKS118]#6	104	97
[pKS118]#7	84	90
[pKS118]#8	91	94

[a]Mitotic stability was determined as described in Materials and methods

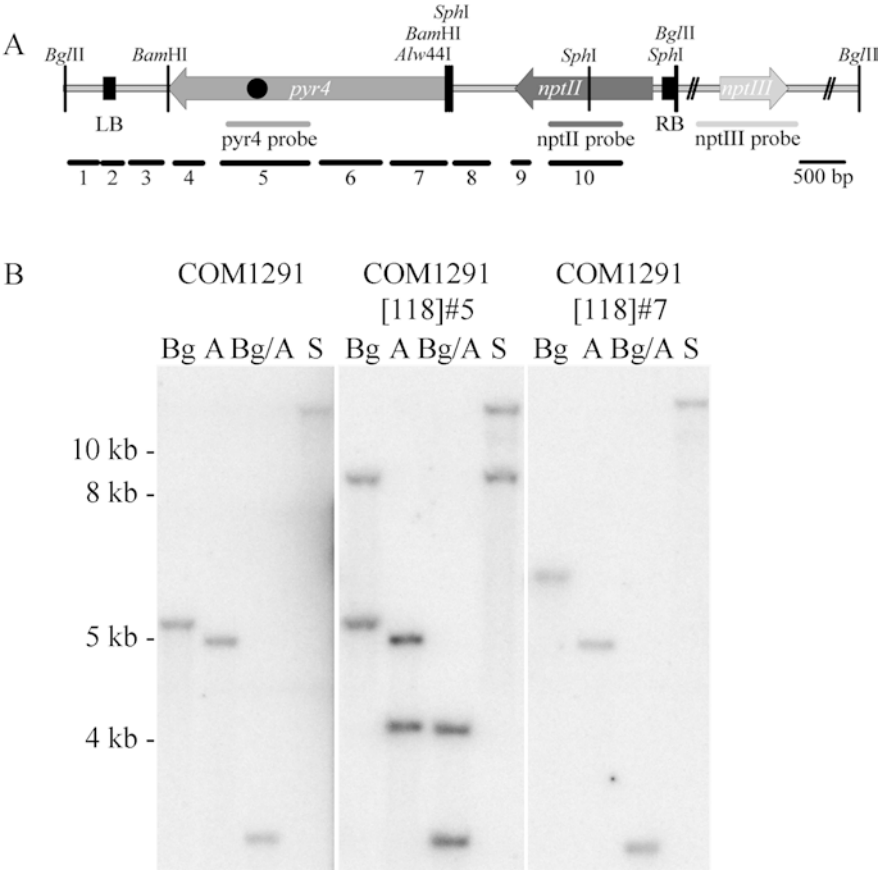

Fig. 2A, B Analysis of T-DNA inserts in transformants obtained by *Agrobacterium*-mediated transformation. **A** T-DNA region of pKS118. LB, left border; RB, right border; *pyr4*, orotidine-5′-monophosphate decarboxylase *R. niveus*, driven by the *R. niveus pyr4* promoter; *nptII*, the neomycin phosphotransferase gene which confers resistance to neomycin and kanamycin, driven by the bacterial nopaline synthetase promoter; *nptIII* encodes 3′5′-aminoglycoside phosphotransferase type III, which confers kanamycin resistance. The *filled circle* indicates the 54-bp deletion in *pyr4* of COM1291. The probes used to analyze the structure of the inserted T-DNA are indicated as follows: 1, pBIN19 backbone; 2, left T-DNA border; 3, pBIN19 T-DNA (M13 ori); 4, *pyr4* terminator; 5, *pyr4* ORF; 6, *pyr4* promoter (−160 to 840 bp) probe; 7, *pyr4* promoter (−990 to 1159 bp); 8, pBIN19 T-DNA (gene III); 9, *nos* terminator; 10, *nptII* probe. **B** Southern analysis of [pKS118] transformants obtained by *Agrobacterium*-mediated transformation, using the *pyr4* probe. COM1291[118]#5 and COM1291[118]#7 are representative type I and type II transformants, respectively. Bg, *Bgl*II; A, *Apa*LI; S, *Sph*I

type I and type II transformants with *Bgl*II, followed by hybridization using short probes covering the entire T-DNA region (Fig. 2A). A signal was found in type II transformants only when probes specific for the *pyr4* region (Table 4) were used; no signals could be detected with probes specific for T-DNA sequences outside the *pyr4* region, indicating, as expected for gene conversion events, that no vector sequences were present in these transformants. For type I transformants a signal was found not only with probes specific for the *pyr4* region, but also with probes specific for the T-DNA of pBIN19, such as the M13 origin of replication (Table 4). However, probes specific for pBIN19 vector sequences outside the T-DNA and probes specific for the *nos* terminator and *nptII* located on the T-DNA were negative (Table 4), indicating that truncation of the T-DNA had occurred between the sequences detected by probes 8 and 9 (Fig. 2A).

Several PCR based strategies, including inverse PCR, TAIL-PCR and vectorette PCR, were used to try to isolate the chromosomal DNA flanking the T-DNA insert. However, no specific PCR product could be obtained (data not shown). Therefore, it was not possible to determine the precise location of the T-DNA breakpoint or to isolate the integration 'hot spot'.

Based on Southern analysis and mitotic stability determinations, we conclude that *Agrobacterium*-mediated transformation of *R. oryzae* results in integration of the introduced DNA into the genome. Detailed analysis of the transformants revealed that the T-DNA had integrated at a single locus (an integration 'hot spot') and that truncation of the T-DNA had occurred.

Discussion

In this study two transformation methods, the $CaCl_2$/PEG method and *Agrobacterium*-mediated transformation, were compared. We show that *Agrobacterium*-mediated transformation of *R. oryzae* results in transformants that are mitotically stable under selective, but also under non-selective, conditions, indicating that

Table 4 Summary of the results of Southern analysis of type I and type II transformants

Probe No.	Probe name	Segment recognized by probe	Fragment sizes detected (kb)		
			COM1291	Type I transformants	Type II transformants
1	pBINp2-pBINp3	pBIN19 vector backbone	-	-	-
2	pBINp4-pBINp5	left T-DNA border	-	-	-
3	pBINp6-pBINp7	pBIN19 T-DNA (M13 ori)	-	8.5	-
4	*pyr* 8-*pyr* 9	*pyr4* terminator	5.5	8.5 + 5.5	6
5	*pyr* 4-*pyr4*ATG	*pyr4* open reading frame	5.5	8.5 + 5.5	6
6	*pyr* 2-*pyr* 10	*pyr4* promoter	5.5	8.5 + 5.5	6
7	*pyr* 11-*pyr* 7	*pyr4* promoter	5.5	8.5 + 5.5	6
8	pBINp8-pBINp9	pBIN19 T-DNA (gene III)	-	8.5	-
9	*nos*	*nos* terminator	-	-	-
10	*nptII*	*nptII*	-	-	-

the transforming DNA has been integrated into the chromosomes. To our knowledge, this is the first demonstration of the successful use of *Agrobacterium*-mediated transformation to obtain stable and integrative transformants in a zygomycete. A recent study by Monfort et al. (2003) also reported the use of *Agrobacterium*-mediated transformation of a zygomycete, *Mucor miehei* (Monfort et al. 2003). However, the *M. miehei* transformants displayed low mitotic stability, even under selective conditions, indicating that the T-DNA had not stably integrated into the genome (Monfort et al. 2003).

Both the $CaCl_2$/PEG and the *Agrobacterium*-mediated transformation systems require the use of protoplasts. For most fungi which have been transformed by *Agrobacterium*-mediated transformation, spores, germlings or fruiting body tissue served as the starting material (de Groot et al. 1998; Abuodeh et al. 2000; Chen et al. 2000). For the transformation of *R. oryzae* with *A. tumefaciens* only protoplasts could be used. No transformants were obtained when spores or germlings were employed as the starting material (data not shown). Differences in cell wall composition among zygomycetes could present a natural barrier to the attachment of *A. tumefaciens* or prevent the transfer of T-DNA. However, *Agrobacterium*-mediated transformation of the zygomycete *M. miehei* using germlings has recently been reported, as mentioned above (Monfort et al. 2003). Unfortunately, the requirement for *R. oryzae* protoplasts as the starting material makes the success of transformation dependent on the number of viable protoplasts available, which varies with the enzyme batch (Goosen et al. 1991; this study). The reproducibility of *Agrobacterium*-mediated transformation of *R. oryzae* could be enhanced if spores or germlings could be used as starting material.

The introduction of circular or linear vector molecules into *R. oryzae* by the $CaCl_2$/PEG method resulted in transformants with multiple, autonomously replicating, vector molecules, and such transformants were mitotically unstable, irrespective of the selection marker used. In contrast to previous experience with *Aspergillus niger* and *A. awamori* (van Gorcom and van den Hondel 1988; Gouka et al. 1995), attempts to force integration into the genome of *R. oryzae* by homologous recombination at the *pyr4* locus using a mutant *pyr4* allele were unsuccessful (data not shown). Rescue of the free plasmids into *E. coli* revealed that about 20% of them had suffered small deletions, indicating possible rearrangement of the DNA in *R. oryzae*, as has been observed in other zygomycetes (Burmester et al. 1990; Arnau et al. 1991; Monfort et al. 2003). Southern analysis of the *R. oryzae* transformants revealed that, in most cases, not only the expected plasmid fragments but also specific additional fragments were observed, most probably indicating partial resistance to digestion by restriction enzymes. This suggests that some modification of the transforming DNA could have occurred in *R. oryzae*. One possible explanation for partial resistance to digestion is methylation, a well-known defence mechanism against foreign DNA in fungi (Goyon and Faugeron 1989; Mooibroek et al. 1990; Birch et al. 1998; Maloisel and Rossignol 1998; Selker 1998). However, analysis of the transformants with methylation-insensitive isoschizomers did not lead to conclusive results. Furthermore, the addition of 5-azacytidine, a potent inhibitor of methylation (Ysraeli and Szyf 1984), did not significantly increase the mitotic stability of the transformants (data not shown). In light of these results, it appears that methylation alone cannot be responsible for the failure to digest the transforming DNA completely. Other uncharacterized genome defence mechanisms might play a role in rearranging the introduced DNA and thus prevent its integration into the chromosomes.

Although the $CaCl_2$/PEG transformation method resulted predominantly in mitotically unstable transformants carrying multiple autonomously replicating plasmid molecules, *Agrobacterium*-mediated transformation resulted in mitotically stable transformants, and chromosomal integration was observed in all the transformants obtained. Even under non-selective conditions, these transformants remained mitotically stable, further indicating that the introduced DNA had integrated into the genome and that integration was stable. Based on these results it can be concluded that the *Agrobacterium*-mediated transformation system can successfully be used to transform the zygomycete *R. oryzae*.

One possible explanation for the successful integration of the DNA introduced by *Agrobacterium*-mediated

transformation is that not naked DNA, but a DNA-protein complex, composed of a linear single stranded DNA molecule and the DNA binding proteins VirD2 and VirE2 is transported and formed in the host cytoplasm (Zupan et al. 2000). The binding of these proteins to the single stranded DNA might prevent the formation of the free double stranded circular DNA molecules necessary for autonomous replication. The presence of the VirD2 protein might also have a beneficial effect on integration, as it is thought to play a role in DNA repair (Bako et al. 2003). Furthermore, single stranded DNA may be a better substrate for integration.

In addition, it has been observed that the host organism itself determines the mode of T-DNA integration by homologous or non-homologous recombination (Bundock et al. 1995; van Attikum et al. 2001). Differences in host organism could explain the observed difference between the maintenance of T-DNA in *R. oryzae* and *M. miehei*. We found that T-DNA integrated into the *R. oryzae* genome is stably maintained, even under non-selective conditions. However, in *M. miehei* the T-DNA is easily lost even under selective conditions (Monfort et al. 2003). Furthermore, the T-DNA copy number in the *M. miehei* transformants was so low that no T-DNA could be detected by Southern analysis. The lack of Southern data makes it impossible to determine whether the T-DNA had integrated into the genome, but the inability to detect the T-DNA suggests that it had not been integrated. This suggestion is supported by the low mitotic stability of the transformants, again indicating that the T-DNA was not stably maintained in *M. miehei*. One important difference between our study and that of Monfort et al. (2003) is that different markers were used to select for transformants. We used the endogenous auxotrophic *pyr4* marker, whereas Monfort et al. (2003) used the exogenous *aph*I marker, which confers kanamycin resistance. As indicated by Monfort et al. (2003), there could be specific genome defence mechanisms present in *M. miehei* which detect the presence of foreign DNA and ensure that it is deleted (Monfort et al. 2003). It is conceivable that such genome defence mechanisms are also present in *R. oryzae*, but are less effective against endogenous DNA, thus allowing its integration. Alternatively, the genome defence mechanisms in *R. oryzae* may be less active than those of *M. miehei* and thus less effective in eliminating foreign DNA.

Southern analysis of the *R. oryzae* transformants obtained by *Agrobacterium*-mediated transformation revealed two types of transformants. Transformants of type II had undergone gene conversion, while type I transformants contained the mutated *pyr4* gene and an extra gene copy, originating from the T-DNA. Detailed analysis of type I transformants showed that not the entire T-DNA was present and that integration had occurred at a single position in the genome. T-DNA truncation could have occurred at any one of various stages during transformation, e.g. T-DNA formation, transport or integration. However, no pseudo-T-DNA borders were present within the T-DNA, making it unlikely that early termination of the formation of the T-DNA could explain the observed truncation. T-DNA truncation may have occurred during or after T-DNA integration. In the latter case, unnecessary sequences could have been spliced out again by a second recombination event. Since the second gene located on the T-DNA is of bacterial origin, it could be that this exogenous DNA is susceptible to the postulated genome defence mechanisms present in *R. oryzae*, which determine its elimination, as has been suggested for the exogenous DNA introduced into *M. miehei* (Monfort et al. 2003). This may imply that *Agrobacterium*-mediated transformation of zygomycetes can only be used for the introduction of endogenous DNA. Future experiments should confirm or contradict this notion and should reveal whether it is possible to use *Agrobacterium*-mediated transformation of *R. oryzae* and other zygomycetes for the introduction of full-length T-DNA including exogenous DNA. Although rearrangement of the T-DNA did occur in *Agrobacterium*-mediated transformation of *R. oryzae*, this method nevertheless has the advantage over the other reported methods for the transformation of zygomycetes that the endogenous part of the T-DNA integrated stably into the genome of *R. oryzae*. With some exceptions, integration of the introduced DNA in fungi belonging to the class of zygomycetes is a rare event (Revuelta and Jayaram 1986; Wöstemeyer et al. 1987; Anaya and Roncero 1991; Burmester 1992; Iturriaga et al. 1992; Benito et al. 1995), which severely hampers the genetic manipulation of this class of fungi. One surprising aspect of the *Agrobacterium*-mediated transformation of *R. oryzae* is the integration of the T-DNA at a single locus. T-DNA integration occurs essentially at random in plants, yeasts and fungi (Feldmann 1991; Koncz et al. 1992; de Groot et al. 1998; Bundock et al. 2002; Michielse et al. 2004a). Based on Southern analysis of the type I transformants using various restriction enzymes it appears that the endogenous *pyr4* locus is intact. Although this represents only indirect evidence, it suggests that the site of integration is not linked to the endogenous locus. Isolation of the integration site and flanking genomic DNA observed in the *R. oryzae* type I transformants could provide further clues as to why integration occurs at this particular locus and could explain why a part of the T-DNA was lost during or after integration. Unfortunately, various attempts to PCR-based strategies to isolate the chromosomal DNA flanking the T-DNA insert were unsuccessful, arguing that integration might have occurred at a site that is difficult to amplify by PCR, e.g. in highly repetitive DNA. Furthermore, efforts to directly clone sequences flanking the integration site by generating a chromosomal sub-library from one of the stable transformants were unsuccessful.

The inefficient integration and/or truncation of foreign DNA delivered to various zygomycetes using $CaCl_2$/PEG, electroporation and biolistic transformation methods (Revuelta and Jayaram 1986; Burmester et al. 1990; Yanai et al. 1990; Anaya and Roncero 1991; Burmester 1992; Gonzalez-Hernandez et al. 1997; Skory 2002), the low mitotic stability of the transformants (Suarez and Eslava 1988; Burmester 1992; Arnau and Stroman 1993; Benito et al. 1995; Horiuchi et al. 1995;Gonzalez-Hernandez et al. 1997) and the truncation of foreign DNA delivered by the *Agrobacterium*-mediated transformation method (this study) all suggest that specific mechanisms for the detection and elimination of foreign DNA exist in zygomycetes which may account for the low rates of success in transforming members of this class of fungi.

Examples of genome defence mechanisms that have been described in fungi include repeat-induced point mutation (RIP) and quelling in *Neurospora crassa* (Cambareri et al. 1991; Romano and Macino 1992), methylation induced premeiotically (MIP) in *Ascobolus immersus* (Rhounim et al. 1992) and transnuclear transcriptional gene silencing in *Phytophtora infestans* (van West et al. 1999). Whether similar defence mechanisms exist in *R. oryzae* and other zygomycetes remains to be resolved. Besides these genome defence mechanisms, methylation or chromatin remodeling, thought to be involved in DNA repair (Morales et al. 2001), could play a role in determining the fate of the introduced DNA in *R. oryzae*. However, we found no evidence that methylation of foreign DNA had occurred in *R. oryzae*.

In conclusion, we have shown that it is possible to obtain stable transformants in which the introduced DNA has integrated into the genome of *R. oryzae*. However, we also observed that a part of the introduced DNA was deleted. Therefore, it seems that *R. oryzae*, and perhaps also other zygomycetes, contain a defence mechanism which recognizes and removes introduced DNA in order to prevent the integration of foreign (exogenous) DNA, thereby protecting the integrity of the genome. The generation of mutants that are less reactive to exogenous DNA could circumvent the difficulties encountered in stably introducing foreign DNA into zygomycetes. Nevertheless, it can be said that *Agrobacterium*-mediated transformation represents a substantial improvement on the existing transformation methods for zygomycetes, since it does result in 100% mitotically stable transformants in which the endogenous DNA has integrated into the genome. Future experiments will reveal if this transformation method can also be used for the introduction of exogenous DNA into zygomycetes.

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