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Agrobacterium-mediated transformation leads to improved gene replacement efficiency in *Aspergillus awamori*

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Abstract

In this study, the efficiency of gene replacement in *Aspergillus awamori* between *Agrobacterium*-mediated transformation and $CaCl_2$/PEG-mediated transformation was compared. For the genes, *pyrG* and *gfaA*, it was found that the homologous recombination frequencies obtained by *Agrobacterium*-mediated transformation were 3- to 6-fold higher than the frequencies obtained with $CaCl_2$/PEG protoplast transformation. For the *pyrG* gene, it was found that *Agrobacterium*-mediated transformation allowed an efficient homologous recombination with shorter DNA flanks than $CaCl_2$/PEG protoplast transformation. Finally, the addition of the dominant *amdS* marker as a second selection marker to the gene replacement cassette led to a further 2-fold enrichment in transformants with gene replacement events, resulting in a gene replacement frequency of 55%. Based on the data it can be concluded that *Agrobacterium*-mediated transformation is an efficient tool for gene replacement and that the *amdS* gene can be successfully used as a second selection marker to select transformants with putative gene replacement.

Index Descriptors: Agrobacterium tumefaciens; Gene targeting; Double marker enrichment technique; Filamentous fungi; *Aspergillus awamori*

1. Introduction

Gene replacement is often used to generate precise deletion mutants to assess a possible function of the deleted gene. In gene replacement experiments a linear fragment, the gene replacement cassette, which consists of a selectable marker gene flanked with DNA fragments homologous to the gene of interest, is introduced into the host where it will integrate either by homologous recombination or by non-homologous recombination (ectopic integration, illegitimate integration).

The integration of foreign DNA sequences into the genome by homologous recombination or ectopic integration seems to be tightly correlated with the dominant pathway of the host to repair double stranded DNA breaks (Schaefer, 2001). Thus, the DNA repair machinery of the host organism mainly determines the efficiency of homologous recombination. In yeasts, like *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, short homologous regions of 50–100 bp are enough to facilitate integration of the gene replacement cassette at the homologous locus, leading to a gene replacement efficiency of 50–100% (Bahler et al., 1998; Wach et al., 1994). However, in filamentous fungi relative long homologous flanks ($\geqslant$1000 bp) are needed in order to achieve gene replacement efficiencies varying from 10 to 30% (Asch and Kinsey, 1990; Hynes, 1996). Besides the homologous recombination efficiency of the host other factors, like the length of the homologous DNA in the gene replacement cassette, the G/C content of these flanks, the transcriptional status of the targeted gene and the chromatin structure determined by the location of

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the targeted gene on the chromosome, also play a role in determining the efficiency of targeted integration (Baudin et al., 1993; Bird and Bradshaw, 1997; Gray and Honigberg, 2001; Hua et al., 1997; Nelson et al., 2003; Versaw and Metzenberg, 1996).

The high gene replacement efficiency in yeasts with short homologous DNA flanks led to the development of PCR-based gene replacement cassettes that avoided laborious cloning (Lau et al., 2002; Lorenz et al., 1995; Wach et al., 1994). Unfortunately, this technique is not applicable for filamentous fungi, since long homologous DNA flanks are needed to obtain a reasonable frequency of homologous recombination. Therefore, other techniques like two-step PCR (Davidson et al., 2002), random transposon mutagenesis into a cosmid (TAGKO) (Hamer et al., 2001) or in vivo recombination in *Escherichia coli* (Chaveroche et al., 2000) have been developed. The last two techniques result in gene disruption or replacement cassettes, which carries large stretches of homologous DNA. It has been shown that both techniques lead to increased homologous recombination efficiency with frequencies of 50–80% (Chaveroche et al., 2000; Hamer et al., 2001).

In addition to the development of methods facilitating the construction of gene replacement vectors with long homologous DNA flanks, methods to identify non-homologous integration events have been developed (Aronson et al., 1994; Liu et al., 2001; Pratt and Aramayo, 2002). To discriminate between homologous and non-homologous recombination events a second dominant selectable marker, which flanks the gene replacement cassette, was added. Upon homologous recombination the second selection marker will be lost, whereas upon non-homologous recombination transformants carrying both selectable markers will be obtained. Applying such a selection strategy led to a 5- to 20-fold enrichment of *Acremonium chrysogenum* and *Neurospora crassa* transformants, respectively, in which recombination had taken place (Aronson et al., 1994; Liu et al., 2001; Pratt and Aramayo, 2002).

Besides conventional transformation methods for filamentous fungi mostly using protoplasts treated with $CaCl_2$/PEG, the *Agrobacterium*-mediated transformation was recently applied with success to fungi (de Groot et al., 1998). This method, derived from plant-cell transformations, is based on the ability of *Agrobacterium tumefaciens* to transfer a part of its DNA (transferred DNA) to eukaryotic cells (reviewed by Zhu et al., 2000; Zupan et al., 2000). The transferred DNA is transported to the host as a single stranded DNA molecule and is accompanied by the DNA-binding proteins VirD2 and VirE2 (Zhu et al., 2000; Zupan et al., 2000). As observed in plants, an intact virulence system of *A. tumefaciens* is necessary for T-DNA transfer to yeasts and filamentous fungi (Bundock et al., 1995; Michielse et al., 2004b). The *Agrobacterium*-mediated transformation method resulted in higher transformation frequencies for most filamentous fungi when compared to conventional methods as well as in the development of transformation systems for fungi, such as *Helminthosporium turcicum* and *Agaricus bisporus* (Lange), which were not possible to transform with the conventional methods (Amey et al., 2002; de Groot et al., 1998; Degefu and Hanif, 2003; Meyer et al., 2003; Mikosch et al., 2001). Furthermore, it has been shown that DNA delivered to yeast and filamentous fungi using the *Agrobacterium*-mediated transformation system promoted homologous recombination in *Kluyveromyces lactis*, *Mycosphaerella graminicola*, *Glarea lozoyensis*, *Verticillium fungicola*, *Trichoderma atroviride*, and *Verticillium dahliae* (Amey et al., 2003; Bundock et al., 1999; Dobinson et al., 2003; Zeilinger, 2003; Zhang et al., 2003; Zwiers and De Waard, 2001). However, a systematic comparison of homologous recombination frequencies obtained by *Agrobacterium*-mediated transformation to those obtained by the conventional ($CaCl_2$/PEG) protoplast transformation method has not been carried out for filamentous fungi.

In this study, we have determined whether the unique way by which *A. tumefaciens* delivers its DNA to its host results in increased gene replacement frequencies compared to the frequencies obtained with the $CaCl_2$/PEG protoplast transformation method. For one gene, *pyrG*, we have systematically analyzed the minimal length of flanking sequences necessary to allow gene replacement in *Aspergillus awamori* using both the *Agrobacterium*-mediated and the $CaCl_2$/PEG protoplast transformation method. The gene replacement cassette consists of the hygromycin selection marker flanked with *pyrG* promoter and terminator sequences of various sizes. The *pyrG* gene was chosen as a target locus, because its replacement can easily be determined based on uridine/uracil dependence of the $pyrG^-$ transformants (Boeke et al., 1984; d'Enfert, 1996). The *gfaA* gene was used as an additional locus. The *gfaA* gene encodes a glucosamine: fructose-6-phosphate aminotransferase required for the synthesis of *N*-acetyl-glucosamine. *GfaA* is an essential gene, but the addition of glucosamine to the growth medium could rescue the deletion strain (Ram et al., 2004). The gene replacement frequencies obtained either with *pyrG* or the *gfaA* replacement constructs were determined and compared to frequencies obtained with the $CaCl_2$/PEG protoplast transformation method. Furthermore, we combined the *Agrobacterium*-mediated transformation with the double marker enrichment technique to discriminate between ectopic and homologous recombination events. As a second selection marker the *amdS* gene was chosen. The *amdS* gene is a heterologous dominant selection marker, which can be used for transformation of several filamentous fungi (Beri and Turner, 1987; Kelly and Hynes, 1985; Penttila et al., 1987; Rodriguez and Yoder, 1987; Yamashiro et al., 1992). The presence of

this gene in ectopic integration events can be detected positively by the ability of the transformants to grow on acetamide as sole carbon and nitrogen source, but its presence can also be negatively selected by the sensitivity of the transformants to the compound fluoro-acetamide (Hynes and Pateman, 1970).

In this paper, we show that the *Agrobacterium*-mediated transformation method is an efficient method to generate gene replacement events in *A. awamori* and that the *amdS* gene can be used as a second selection marker to enrich the pool of transformants, which have undergone homologous recombination.

2. Materials and methods

2.1. Strains and growth conditions

Aspergillus awamori CBS115.52 (CBS, The Netherlands) was used as a recipient strain for transformation. *A. tumefaciens* LBA1100 (pAL1100ΔT-DNA, Δ*tra*, Δ*occ*) (Beijersbergen et al., 1992) was used for *Agrobacterium*-mediated transformation and was grown in LB medium (Sambrook et al., 1989) containing spectinomycin (250 μg/ml) and kanamycin (100 μg/ml) at 28 °C. Introduction of the plasmids into LBA1100 was performed as described by Mattanovich et al. (1989). *Escherichia coli* XL1-Blue (Stratagene) was used for construction, propagation, and amplification of plasmids and was grown in LB medium at 37 °C containing either ampicillin (50 μg/ml) or kanamycin (25 μg/ml) dependent on the resistance marker of the plasmid used.

2.2. Sequencing pyrG and gfaA of A. awamori

The promoter and terminator region of *A. awamori pyrG* (orotidine-5′-monophosphate decarboxylase) was sequenced using the plasmids pAw4-1, pAw4-2, and pAw4-4, carrying a genomic insert of the *pyrG* region (Gouka et al., 1995) as a template. The nucleotide sequence of *pyrG* from *A. awamori* is deposited in GenBank under Accession No. AY530810.

The promoter and terminator region of *A. awamori gfaA* (glucosamine:fructose-6-phosphate aminotransferase) was isolated by PCR using primers based on the *Aspergillus niger gfaA* sequence (A.F.J. Ram, M. Arentshorst, and R. Damveld, unpublished results). The primers pAwgfaAP3 (5′-gttgatgtagccgaaaatgcc-3′) and pAwgfaAP10 (5′-tcccactcaattatctcggttc-3′) were used for the isolation of the promoter region and pAwgfaAP5 (5′-gagagggtctcaacgtcga-3′) and pAwgfaAP8 (5′-atgaccggcactcccgtat-3′) were used for the isolation of the terminator region. Subsequently, the PCR products corresponding to the promoter and terminator region of *gfaA* were ligated into pGEMT-easy (Promega) resulting in pGEMT-PgfaA and pGEMT-TgfaA, respectively. Both plasmids were sequenced using the primers pAwgfaAP3, pAwgfaAP5, pAwgfaAP8, and pAwgfaAP10. The *gfaA* promoter and terminator sequence of *A. awamori* is deposited in GenBank under Accession Nos. AY530808 and AY530809, respectively.

Sequencing of *pyrG* and *gfaA* was carried out on Perkin–Elmer ABI PRISM 310 using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

2.3. Construction of the pyrG replacement constructs

A fusion PCR approach was used to construct pAwKO1000 consisting of the *E. coli hph* gene (conferring to hygromycin resistance) under the control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter flanked by 1110 bp *pyrG* promoter region and 1011 *pyrG* terminator region (Fig. 1). Based on the *pyrG* sequence, the primers pAwKOP1 and pAwKOP2, and pAwKOP5 and pAwKOP6 (Table 1), containing convenient restriction cloning sites, were designed and used in a PCR to obtain an 1110 bp promoter and 1011 bp terminator region, respectively. The hygromycin resistance cassette was amplified using the primers pAwKOP3 and pAwKOP4 (Table 1) and pAN7.1 (Punt et al., 1987) as a template. The first fusion PCR was performed using the primers pAwKOP1 and pAwKOP4, and as a template, the PCR products

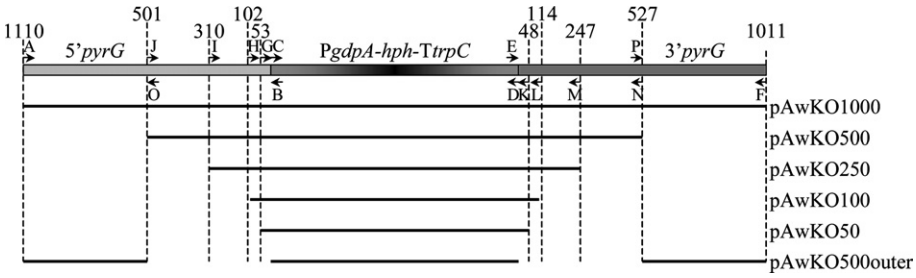

Fig. 1. Schematic overview of the *pyrG* gene replacement constructs. The numbers above the dotted lines indicate the length of the sequences flanking the hygromycin resistance cassette. The construct name is listed to the right of the construct. The PCR primers used to make the gene replacement constructs are presented as arrows above and below the depicted construct and designated by letters (see Table 1).

Table 1
Primers used for making *pyrG* and *gfaA* gene replacement constructs

Primer	Name	Sequence (5′–3′)
A	pAwKOP1	GGAAGATCTCCCACCATGCTTACTCTGAC
B	pAwKOP2	CGACTTTGATGGTCGTTGTAGGTCGGTGGAGGGGTTAATG
C	pAwKOP3	CTACAACGACCATCAAAGTCG
D	pAwKOP4	TCGAGTGGAGATGTGGAGTG
E	pAwKOP5	CACTCCACATCTCCACTCGAGAAATGCAACTTGCCGCAACG
F	pAwKOP6	GGGGTACCGGCAGCGGGGAAGAAGGG
G	pAwKOP7	GGAAGATCTGCTACTCTCATTGCACAAGC
H	pAwKOP8	GGAAGATCTCTGCACATCTCTGGATCTAC
I	pAwKOP9	GGAAGATCTGGCACATTCAATGGAAGGAAC
J	pAwKOP10	GGAAGATCTGACTTCGTGGGTGTGATTG
K	pAwKOP11	GGGGTACCGTGCTCGACATTAGTTTGTATGC
L	pAwKOP12	GGGGTACCGTGCCACACATAATAGCCTG
M	pAwKOP13	GGGGTACCGTCAGCATTGCTTATCTGCG
N	pAwKOP14	GGGGTACCAGGCGATAGGACAAGGATGG
O	pAwKOP10rev	CGACTTTGATGGTCGTTGTAGCAATCACACCCACGAAGTC
P	pAwKOP14rev	CACTCCACATCTCCACTCGACCATCCTTGTCCTATCGCCT
	pAwgfaA15	CCCAAGCTTAGGGTCTCAACGTCGATTTCC
	pAwgfaA16	GGGGTACCGCCTTTGGCAGCTTCAATCTG
	pAwgfaA17	GAAGATCTTATCTCGGTTCCCATGGGC
	pAwgfaA18	CCGCTCGAGTCGTTTGCCGCTCCAAGA
	pAmdS1	GCTCTAGAATCTACGCCAGGACCG
	pAmdS2	GAAATCGGGCATCCTTTCAGAG
	pAmdS3	CTCTGAAAGGATGCCCGATTTC
	pAmdS4	CATAAGGTGGCGTTGTTACATC

Restriction sites are underlined and altered nucleotide sequences are shown in bold.

corresponding to the *pyrG* promoter region and hygromycin cassette. The second fusion PCR was performed using primers pAwKOP1 and pAwKOP6, and as a template, the PCR products corresponding to the *pyrG* promoter region fused to the hygromycin cassette and the *pyrG* terminator region. The PCR product was cloned in pGEMT-easy (Promega), resulting in pGEM-KO1000. Subsequently, pGEM-KO1000 was digested with *Bgl*II and *Kpn*I and ligated into *Bam*HI and *Kpn*I digested pSDM14 (Offringa et al., 1990), resulting in pAwKO1000. The constructs containing shorter *pyrG* flanks (Fig. 1) were constructed by PCR using the primers listed in Table 1 and pAwKO1000 as a template. The PCR products obtained were ligated into pGEMT-easy, resulting in pGEM-KO500, 250, 100, 50, 1000–250, 250–1000, 1000–100, and 100–1000. Each construct was, subsequently, digested with *Bgl*II and *Kpn*I and ligated into pSDM14 digested with *Bam*HI and *Kpn*I, resulting in pAwKO500, 250, 100, 50, 1000–250, 250–1000, 1000–100, and 100–1000, respectively. A similar fusion PCR approach, as described above, was also used for the construction of pAwKO500outer (Fig. 1). For the amplification of the hygromycin resistance cassette the primers pAwKOP3 and pAwKOP4 and, as a template, pAN7.1 were used. The promoter region was amplified with primers pAwKOP1 and pAwKOP10rev. The terminator region was amplified using primers pAwKOP14rev and pAwKOP6. The final fusion PCR product was ligated in pGEMT-easy, resulting in pGEM-500outer. A *Bgl*II–*Kpn*I fragment consisting of the hygromycin resistance cassette flanked with 609 bp promoter region and 484 bp terminator region was ligated into pSDM14 digested with *Bam*HI and *Kpn*I, resulting in pAwKO500outer.

2.4. Construction of the gfaA replacement construct

The gene replacement construct, pAwKOgfaA, was constructed in a two-step approach. To isolate a 980 bp *gfaA* promoter region a PCR was performed on pGEM–PgfaA with the primers pAwgfaA17 and pAwgfaA18 containing a *Bgl*II and an *Xho*I restriction site, respectively. A PCR was also performed on pGEM-TgfaA in order to isolate a 1012 bp *gfaA* terminator region with primers pAwgfaA15 and pAwgfaA16 containing a *Hin*dIII and *Kpn*I restriction site, respectively. The *Hin*dIII–*Kpn*I digested terminator region was ligated in a three-point ligation with an *Xho*I–*Hin*dIII fragment of pAN7.1, corresponding to the hygromycin cassette into pBluescript SKII (Stratagene) digested with *Xho*I and *Kpn*I, resulting in pBlue-HYG-TgfaA. An *Xho*I–*Kpn*I fragment was isolated from pBlue-HYG-TgfaA and ligated in a three-point ligation with the *Bgl*II–*Xho*I digested PCR product of the promoter region into pUC21 (Vieira and Messing, 1982) digested with *Bgl*II and *Kpn*I, resulting in pUC-PgfaA-HYG-TgfaA. Subsequently, a *Bgl*II–*Kpn*I fragment from pUC-PgfaA-HYG-TgfaA was ligated into pSDM14 digested with *Bam*HI and *Kpn*I, resulting in pAwKOgfaA.

2.5. Construction of the double selection marker constructs

To inactivate the *Bgl*II and *Bam*HI restriction sites present in the *amdS* expression cassette, a fusion-PCR approach was used to amplify a part of the *amdS* gene, resulting in a nucleotide insertion 644 nucleotides upstream of the start codon and a G/C substitution at position 129. A fusion PCR product consisting of the PCR products obtained with pAmdS1–pAmdS3 and pAmdS2–pAmdS4 was amplified using primers pAmdS1 and pAmdS4. Subsequently, the fusion PCR product was ligated into pGEMT-easy resulting in pGEMAmdSΔBB. A plasmid containing the entire *amdS* cassette was obtained using a three-point ligation. An *Xba*I–*Bgl*II fragment of pGEMAmdSΔBB and a *Bgl*II–*Xba*I fragment of p3SR2 (Corrick et al., 1987) was ligated into pBluescript SKII (Stratagene) digested with *Xba*I, resulting in pBlueAmdS. The *Xba*I *amdS*-fragment of pBlueAmdS was blunted-ended and ligated into pSDM14 previously digested with *Sal*I and blunted-ended, resulting in pSDMAmdSΔBB. A *Bgl*II–*Kpn*I fragment of pAwKO1000 was ligated into pSDM-AmdSΔBB digested with *Bam*HI and *Kpn*I, resulting in LB-AmdS-KO1000.

RB-AmdS-KO1000 was constructed by ligation of the *Xba*I *amdS*-fragment of pSDMAmdSΔBB previously blunted-ended into pAwKO1000 digested with *Hpa*I.

2.6. Agrobacterium-mediated transformation of A. awamori

Agrobacterium-mediated transformation of *A. awamori* with *Agrobacterium* strains carrying the various gene replacement constructs was performed as described by de Groot et al. (1998) with minor adjustments (Michielse et al., 2004b). Co-cultivation was performed on IM (Bundock et al., 1995) supplemented with 2 mM uridine or 4 mg/ml glucosamine. Transformants were selected on MM (Punt and van den Hondel, 1992) supplemented with 200 μM cefotaxim, 100 μg/ml hygromycin, and 10 mM uridine or on MM supplemented with 200 μM cefotaxim, 100 μg/ml hygromycin, and 20 mg/ml glucosamine depending on the gene replacement construct used.

2.7. $CaCl_2$/PEG protoplast transformation of A. awamori

A. awamori was transformed with a linear *Bgl*II–*Kpn*I DNA fragment corresponding to the various *pyrG* and *gfaA* gene replacement cassettes using $CaCl_2$/PEG protoplast transformation as described by de Graaff (1989) with the following modifications. Protoplasts were obtained after incubation for two hours at 37 °C in 10 ml SMC (de Graaff, 1989) with 20 mg/ml lysing enzyme (Sigma) per 1 g mycelium. Transformants were selected on MM (Punt and van den Hondel, 1992) supplemented with 100 μg/ml hygromycin and 10 mM uridine or on MM supplemented with 100 μg/ml hygromycin and 20 mg/ml glucosamine depending on the gene replacement construct used.

2.8. DNA isolation and Southern analysis

Fungal chromosomal DNA isolation and Southern analysis was performed as described by Kolar et al. (1988) and by Michielse et al. (2004a), respectively. Genomic DNA was digested with *Nco*I and probed with either a *pyrG* probe obtained by PCR using the primers pAwKOP5–pAwKOP13 and, as a template, pAw4-4 (Gouka et al., 1995), or with a 3.1 kb *Xho*I–*Hin*dIII fragment of plasmid pAN7.1 (Punt et al., 1987) (HYG probe), corresponding to the hygromycin cassette, or with a 2.6 kb *Xba*I fragment of plasmid p3SR2 (Corrick et al., 1987), corresponding to the *AmdS* expression cassette (*amdS* probe) (Fig. 3A).

3. Results

Comparison of gene replacement frequencies between $CaCl_2$/PEG and *Agrobacterium*-mediated transformation.

The gene replacement efficiency in *Agrobacterium*-mediated transformation (AMT) was determined by transforming *A. awamori* to hygromycin resistance with different *pyrG* deletion constructs. These constructs consist of the hygromycin resistance cassette flanked with varying lengths of promoter and terminator sequence homologous to *pyrG* (Fig. 1). Primary transformants were selected on minimal medium containing hygromycin and uridine. Subsequently, the *pyrG* phenotype was determined by growing the hygromycin resistant colonies on minimal medium with and without uridine. The gene replacement (GR) frequency was defined as the number of *pyrG*$^-$ colonies divided by the total number of hygromycin resistant colonies grown on minimal medium supplemented with uridine. The GR frequency was determined for each construct and compared to GR frequencies obtained with the $CaCl_2$/PEG protoplast transformation method.

The GR frequency obtained with the AMT was consistently higher than the GR frequency obtained with the $CaCl_2$/PEG protoplast transformation method for constructs with the following *pyrG* flank sizes: 1000, 500, and 250 bp (Table 2). With 250 bp flanks it was still possible to obtain *pyrG*$^-$ colonies with the *Agrobacterium*-mediated transformation, however, these flanks were too short to obtain *pyrG*$^-$ colonies in $CaCl_2$/PEG protoplast transformation. The constructs with 100 and 50 bp *pyrG* flanks did not result in any *pyrG*$^-$ colonies

Table 2
Comparison of gene replacement frequencies using *Agrobacterium*-mediated transformation and $CaCl_2$/PEG protoplast transformation

pyrG flanks (bp)	Replacement efficiency *Agrobacterium* (%)	Replacement efficiency $CaCl_2$/PEG (%)
1000	29 ($n^a = 718$)	10 ($n = 121$)
500	5 ($n = 285$)	2 ($n = 150$)
250	1 ($n = 457$)	0 ($n = 141$)
100	0 ($n = 318$)	n.d.[b]
50	0 ($n = 120$)	n.d.
500outer	34 ($n = 200$)	n.d.

[a] n, number of transformants analyzed.
[b] n.d., not determined.

with the AMT or $CaCl_2$/PEG protoplast method (Table 2). Even direct selection for *pyrG*$^-$ transformants on minimal medium containing 5-fluoro-orotic acid (FOA) (Boeke et al., 1984) with either transformation method did not result in FOA resistant colonies for the constructs with 100 and 50 bp *pyrG* flanks (data not shown). Apparently, these flanks are too short to obtain homologous recombination at the *pyrG* locus in *A. awamori*. The *pyrG* phenotype of several transformants obtained in the *Agrobacterium*-mediated transformation with T-DNA carrying homology to *pyrG* of 1000, 500 and 250 bp flanks was confirmed by Southern hybridization. Transformants corresponding to a *pyrG* gene replacement event have a single *Nco*I restriction fragment shifted to 2659 bp (Fig. 2, lane 1) compared to wild type 3091 bp fragment (Fig. 2, lane WT). Transformants resulting from the integration of the T-DNA at an ectopic location displayed at least two *Nco*I restriction fragments (Fig. 2, lanes 3, 6, and 9) corresponding to the wild type *pyrG* locus (3091 bp) and the *pyrG* T-DNA (various sizes). Patterns corresponding to a *pyrG* gene replacement were observed for all the *pyrG*$^-$ transformants analyzed (Fig. 2). A decrease in the GR frequency was found from 29 to 5% in AMT when the *pyrG* flank

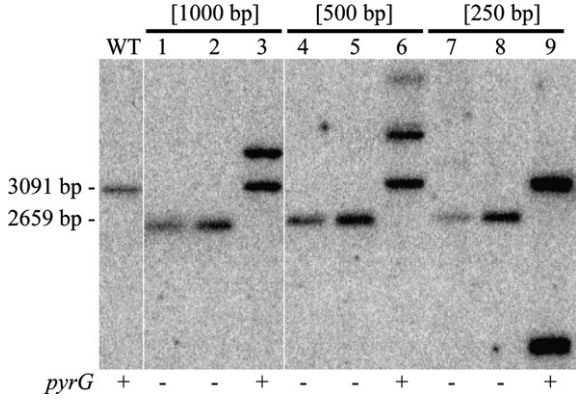

Fig. 2. Southern analysis *pyrG* knock out transformants (+ and − represents *pyrG* phenotype). Genomic DNA of transformants obtained with pAwKO1000 (lanes 1, 2, and 3), pAwKO500 (lanes 4, 5, and 6), or pAWKO250 (lanes 7, 8, and 9) was digested with *Nco*I and probed with *pyrG* probe.

size was reduced from 1000 to 500 bp (Table 2). To determine whether the decrease in GR frequency was simply caused by the shorter flanks or that the sequence itself might influence the GR-frequency, a new construct was made consisting of the hygromycin resistance cassette flanked with the outer 500 bp *pyrG* flanks, pAwKO500outer (Fig. 1). A GR frequency of 34% was obtained when this construct was used to transform *A. awamori* with the AMT method (Table 2). The GR frequency obtained with this construct is higher than the frequency obtained with pAwKO500 (inner 500 bp flanks, 5%) and comparable to the recombination frequency obtained with the 1000 bp *pyrG* flanks (29%), indicating that these outer 500 bp are beneficial for homologous recombination (HR) or that the inner 500 bp are detrimental for HR. Furthermore, it indicates that GR frequencies are not only determined by the length of the flanks. In addition to systematically reducing the size of homologous flanks, we also determined the GR frequencies of gene replacement cassettes carrying *pyrG* homologous flanks of different sizes at the right and left borders. The vectors pAwKO1000-250 and pAwKO1000-100 contain the same large *pyrG* promoter left flank (1000 bp) and different short *pyrG* terminator right flanks of 250 or 100 bp. GR frequencies of 20 and 6% were obtained with pAwKO1000-250 and pAwKO1000-100 (Table 3), indicating that a short flank on one side of the replacement cassette and a longer one on the other side can still lead to efficient gene replacement.

Homologous recombination was shown to be locus dependent (Bird and Bradshaw, 1997) and therefore a second gene, glucosamine:fructose-6-phosphate aminotransferase (*gfaA*), was used to assess the gene replacement efficiency in *Agrobacterium*-mediated transformation and compared to the efficiency obtained in $CaCl_2$/PEG protoplast transformation. A *gfaA* gene replacement cassette, composed of the hygromycin resistance cassette flanked with 980 bp promoter and 1012 bp terminator sequences of *gfaA*, was introduced by transformation into *A. awamori*. The *gfaA* phenotype was determined by growing the hygromycin resistant colonies on minimal medium with or without glucosamine. The GR frequency of *gfaA* was determined by dividing the number of *gfaA*$^-$ transformants by the total number

Table 3
Gene replacement frequencies obtained using T-DNA gene replacement cassettes with *pyrG* homologous sequences of different sizes.

pyrG flanks (LB-RB bp)	Replacement efficiency *Agrobacterium*(%)
1000–250	20 ($n^a = 200$)
250–1000	4 ($n = 200$)
1000–100	6 ($n = 200$)
100–1000	1 ($n = 200$)

[a] *n*, number of transformants analyzed.

of hygromycin resistant colonies. A GR frequency of 50% (number of transformants analyzed (n) = 298) was found in AMT, whereas a GR frequency of 8% (n = 111) was found in $CaCl_2$/PEG protoplast transformation. Thus, a higher GR frequency was obtained when the *Agrobacterium*-mediated transformation was compared to the $CaCl_2$/PEG protoplast transformation for the replacement of *gfaA*.

Based on these results, we conclude that introduction of the gene replacement cassette by *Agrobacterium*-mediated transformation results in a higher gene replacement frequency compared to the $CaCl_2$/PEG-mediated transformation.

3.1. The amds gene as a second selection marker

To enrich the pool of gene replacement transformants and thereby reducing the number of primary transformants to be screened to isolate gene replacement transformants, the use of the *amdS* gene as a second selection marker was assessed. Two constructs were made both containing the hygromycin selection marker flanked with 1000 bp *pyrG* flanks and at either the left or the right border the *amdS* cassette (LB-AmdS-KO1000 and KO1000-AmdS-RB, Fig. 3). Both constructs were used in AMT. Transformants were first selected on minimal medium supplemented with hygromycin and uridine. Subsequently, the *pyrG* phenotype and GR frequencies were determined.

It was found that the addition of the *amdS* gene to the *pyrG* promoter flank (LB) resulted in a GR frequency of 6%, whereas addition of the *amdS* gene to the *pyrG* terminator flank (RB) resulted in a GR frequency of 8% (Table 4). It should be noted that addition of the *amdS* gene resulted in a lower GR compared to the value (29%) obtained with the pAwKO1000 construct containing the 1000 bp homologous flanks without the *amdS* gene (Tables 2 and 4). All *pyrG*$^-$ transformants obtained did hardly grow on medium containing acetamide, indicating that a gene replacement event always led to a loss of the second selection marker. The same collection of transformants was analyzed by replica-plating to examine whether pre-selection on agar plates containing acetamide would result in an enrichment of gene replacement transformants. Hygromycin resistant colonies were tested for their ability to grow on agar plates containing acetamide or uracil. Selection of hygromycin resistant transformants with an *amdS*$^-$ phenotype increased the number of transformants resulting from gene replacement by 5- or 7-fold when the *amdS* gene was located at the LB or at the RB, respectively (Table 4). Furthermore, it was found that addition of the *amdS* marker to the terminator region (RB) of pAWKO1000 (RB-AmdS-KO1000) enriched the pool of gene replacement transformants 2-fold (Tables 2 and 4). Counter-selection of the *amdS* gene was also attempted by

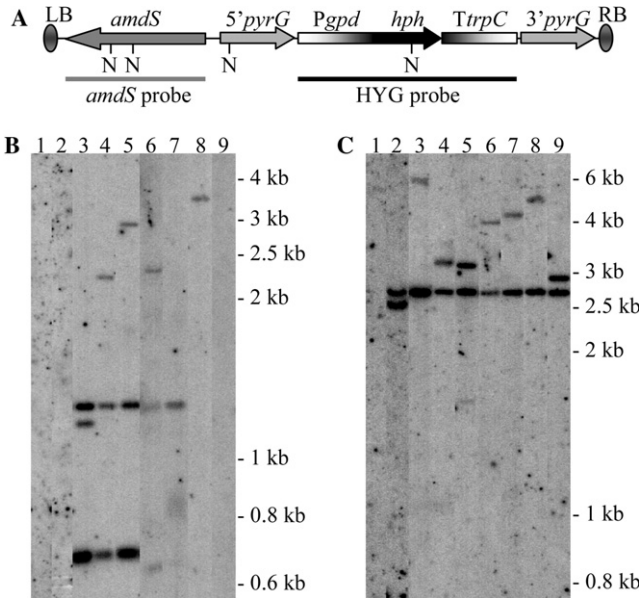

Fig. 3. (A) T-DNA region of LB-AmdS-KO1000. LB, left border. RB, right border. *amdS*, *A. nidulans* acetamidase enzyme. *pyrG*, *A. awamori* orotidine-5′-monophosphate decarboxylase. P*gpd*, *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter. *hph*, hygromycin resistance gene. T*trpC*, *A. nidulans trpC* terminator. N, *Nco*I restriction site. Southern analysis of LB-AmdS-KO1000 transformants. Genomic DNA digested by *Nco*I and probed with AmdS probe (B) and HYG probe (C). 1, wild type; 2, [LB-AmdS-KO1000*pyrG*$^-$/*AmdS*$^-$]#2; 3, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^+$]#6; 4, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^+$]#7; 5, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^+$]#9; 6, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^-$]#8; 7, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^-$]#10; 8, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^-$]#2; and 9, [LB-AmdS-KO1000*pyrG*$^+$/*AmdS*$^-$]#9.

selecting the transformants directly on plates containing fluoro-acetamide. The addition of fluoro-acetamide to the transformation plates resulted in very small colonies, which made it very difficult to discriminate between *amdS*$^+$ and *amdS*$^-$ transformants (data not shown). This result indicates that wild type *A. awamori* contains some acetamidase-like activity and this complicates the selection of *amdS*$^-$ transformants directly on agar plates containing fluoro-acetamide.

Transformants, which contain an ectopic integration of the gene replacement construct, are expected to be *pyrG* positive and are expected to grow on acetamide as a sole nitrogen source. However, among *pyrG*$^+$ transformants with an ectopic T-DNA integration, we identified both *pyrG*$^+$/*AmdS*$^+$ and *pyrG*$^+$/*amdS*$^-$ transformants, indicating that the *amdS* gene from the gene replacement cassette is not expressed or has been lost during integration. The different classes of transformants obtained with LB-AmdS-KO1000 were analyzed at the DNA level for the presence of the *amdS* marker. Genomic DNA was digested with *Nco*I and probed with the hygromycin and the *amdS* gene (Fig. 3). As expected, analysis of a *pyrG*$^-$/*AmdS*$^-$ transformant probed with hygromycin revealed the expected fragments (Fig. 3C,

Table 4
Gene replacement frequencies obtained using the *amdS* gene as a second selection marker

Construct	Number of *hph*$^+$ colonies	Number of *amdS*$^-$ colonies	Number of *pyrG*$^-$ colonies	GR[a] (%)	GRpre[b] (%)
LB-AmdS-KO1000	1737	366	104	6	28
KO1000-AmdS-RB	1199	182	100	8	55

[a] GR is the gene replacement frequency obtained without *amdS* pre-selection, which was determined by dividing the number of *pyrG*$^-$ transformants by the total number of hygromycin resistant transformants.

[b] GRpre is the gene replacement frequency obtained with *amdS* pre-selection, which was determined by dividing the number of *pyrG*$^-$ transformants by the number of *amdS*$^-$ transformants.

lane 2, 2600 and 2469 bp), confirming gene replacement at the *pyrG* locus. This transformant did not contain the *amdS* gene (Fig. 3B, lane 2). All the *pyrG*$^+$ transformants, both *amdS*$^+$ and *amdS*$^-$, revealed the expected fragments when probed with the hygromycin cassette. An internal hygromycin fragment (2469 bp) and a second fragment corresponding to the remaining of the hygromycin cassette flanked with chromosomal DNA (2507 bp). This second fragment is different in length in all transformants analyzed, indicating that T-DNA integration occurred randomly (Fig. 3C). Genomic DNA of the *pyrG*$^+$/*AmdS*$^+$ transformants probed with the *amdS* gene showed the expected fragments (1269 $\geqslant$ 998 and 675 bp), confirming the presence of the entire *amdS* gene (Fig. 3B, lanes 3, 4, and 5). Southern analysis of the *pyrG*$^+$/*amdS*$^-$ transformants revealed three different classes of transformants. In the first class the entire T-DNA including the complete *amdS* gene is present (Fig. 3B, lane 6). However, based on the growth of this class of transformants on medium containing acetamide, it can be concluded that the *amdS* gene is present, but is not expressed at high enough levels to result in growth on acetamide containing agar plates. The second class of *pyrG*$^+$/*amdS*$^-$ transformants contains only part of the *amdS* gene (Fig. 3B, lanes 7 and 8). The third class of *pyrG*$^+$/*amdS*$^-$ transformants does not contain the *amdS* gene at all (Fig. 3B, lane 9). These two types of transformants could result from LB truncation during T-DNA integration.

In conclusion, it was found that deletion of the *pyrG* gene by gene replacement was always correlated with the loss of the *amdS* marker. The results also show that the *amdS* gene can successfully be used to enrich the pool of putative gene replacement transformants.

4. Discussion

Agrobacterium-mediated transformation was shown to be suitable for gene replacement in various filamentous fungi (Amey et al., 2003; Dobinson et al., 2003; Zeilinger, 2003; Zhang et al., 2003; Zwiers and De Waard, 2001). In this study, we have compared the frequency of gene replacement obtained using *Agrobacterium*-mediated transformation to protoplast $CaCl_2$/PEG transformation. In addition, we have examined the effect of the length of homologous DNA flanks on the gene replacement frequency and improved the selection of the transformants resulting from homologous recombination using a second selection marker.

The gene replacement cassettes used in this study have been constructed in such a way that replacement of the *pyrG* locus could be achieved. It was found that introduction of the gene replacement cassette, which carried 1000 bp homologous *pyrG* flanks, by *Agrobacterium*-mediated transformation resulted in a 3-fold increase in the GR frequency in *A. awamori* compared to the GR frequency obtained by $CaCl_2$/PEG protoplast transformation. Since gene targeting has been found to be locus dependent (Bird and Bradshaw, 1997), we also determined gene replacement efficiency of a second gene, *gfaA*. In these experiments gene replacement frequency at the *gfaA* locus increased 6-fold when DNA was delivered to *A. awamori* by AMT compared to the $CaCl_2$/PEG protoplast transformation, indicating that AMT resulted consistently in higher GR frequencies compared to the frequencies obtained with the $CaCl_2$/PEG protoplast transformation method. In a previous study, it was also observed that the use of AMT led to higher GR frequencies in *K. lactis* compared to frequencies obtained after electroporation (Bundock et al., 1999). This suggests that the high gene replacement frequencies found in *Agrobacterium*-mediated transformation of fungi and yeasts are a result of the way *A. tumefaciens* delivers its DNA to the host and are not due to locus specificity of the targeted gene or host dependent. One explanation for the higher frequency of homologous recombination obtained with AMT could be due to the fact that a single stranded DNA–protein complex is delivered to the host, rather than a double stranded DNA molecule, as in $CaCl_2$/PEG protoplast transformation. The DNA–protein complex is composed of a single stranded DNA molecule and the virulence proteins VirD2 and VirE2 (Zupan et al., 2000). The single stranded nature of the transferred DNA may promote homologous recombination. It has been shown in *Streptomyces* species and *S. cerevisiae* that single stranded DNA (ssDNA) is a preferred substrate for integration and homologous recombination, respectively (Hilleman et al., 1991; Simon and Moore, 1987). However, a putative role of the single

stranded DNA-binding virulence proteins, VirD2 and VirE2, in homologous recombination cannot be ruled out. The presence of these proteins could result in more intact DNA molecules reaching the nucleus than in $CaCl_2$/PEG protoplast transformation, as the binding of these proteins to the ssDNA has been shown to protect the DNA against nucleases and targets the DNA–protein complex to the nucleus (Christie et al., 1988; Durrenberger et al., 1989; Rossi et al., 1993; Rossi et al., 1996). The presence of the VirD2 protein may also have a beneficial effect on DNA integration as it is thought to play a role in DNA repair (Bako et al., 2003).

The homologous DNA flank sizes of the *pyrG* gene replacement cassettte was stepwise reduced in order to determine the minimal flank size necessary to obtain gene replacement at a reasonable frequency. As expected, systematic shortening of the flanks, which is known to have an influence on homologous recombination efficiencies (Bird and Bradshaw, 1997; Gray and Honigberg, 2001; Nelson et al., 2003), led to a decrease in the GR frequency. However, in all cases, the gene replacement efficiencies were consistently higher when DNA was delivered by AMT compared to $CaCl_2$/PEG protoplast transformation. These experiments also showed that when the gene replacement cassette is delivered by AMT shorter homologous DNA flanks could be used to obtain transformants, which have undergone homologous recombination. A difference in GR was found when the 1000 bp homologous DNA flanks were cut in two, resulting in gene replacement cassettes with either 500 bp inner or 500 bp outer homologous DNA flanks. These experiments indicate that not only the length, but also the sequence itself influences GR frequency. It also indicates that increasing the flank sizes does not necessarily mean that the GR frequency will increase. In fact, a comparable GR frequency was found with the outer 500 bp homologous flanks (34%) versus the 1000 bp flanks (29%). Sequence analysis of the 500 inner and outer bps flanks did not reveal the presence of any motifs that could be beneficial for HR, like large G/C stretches. The G/C content, which is known to have an influence on HR (Gray and Honigberg, 2001), did not account for the observed difference, as the G/C content of both flanks was comparable (ranging from 46 to 53%). It could be that the chromatin structure of the targeted gene could play a role in the observed difference.

Homologous DNA flanks of 100 and 50 bp were too short to obtain GR in *A. awamori* at the *pyrG* locus with either transformation method. However, with different sized flanks, 1000 bp on one side of the selection marker and 100 on the other side, it was possible to obtain GR by AMT at the *pyrG* locus. This presents the possibility of generating a gene replacement cassette with a short flanking sequence incorporated into a single primer on one side and a longer, PCR generated sequence on the other side, thereby facilitating the generation of gene replacement cassettes for filamentous fungi. This could be especially useful if the genome sequence for a given gene is limited. Alternatively, the high efficiency of GR recombination by AMT could be combined with the in vivo recombination methods, such as TAGKO, to enable gene targeting for organisms in which the homologous recombination efficiency is very low.

To reduce the number of primary transformants to be screened for gene replacement events, a double marker enrichment technique in combination with AMT was applied. The *amdS* gene was chosen as second selection marker, because its presence can both be positively and negatively selected (Hynes and Pateman, 1970). Furthermore, since many fungal species do exhibit no or low acetamidase activity (Goosen et al., 1991), the *amdS* gene as a second selection marker might be applicable to a wide variety of fungi. In this study, the presence of the *amdS* gene was determined by replica plating of the transformants onto agar plates containing acetamide. Although the addition of the *amdS* selection marker to the gene replacement cassette led to 5-fold decrease in the gene replacement efficiency, pre-selecting the primary transformants on acetamide containing medium, reduced the total number of transformants which needed to be screened to identify a gene replacement event 2-fold when the *amdS* gene was placed at the terminator region. Placement of the *amdS* gene at the promoter region (LB) of the gene replacement cassette did not result in a significance difference in the final GR frequency. Thus, placement of the *amdS* gene at the terminator region (RB) resulted in more $pyrG^-$ and/or in less false negative *amdS* ($pyrG^+/amdS^-$) transformants. One putative explanation could be that in AMT the *amdS* gene is better preserved when located at the right T-DNA border (RB) than at the LB, thereby, resulting in less false negative $pyrG^+/amdS^-$ transformants. It is known that the LB is more sensitive to truncation (Rossi et al., 1996), whereas, the RB is better preserved due to the covalent binding of the VirD2 protein to the 5′ end of the transferred DNA (Durrenberger et al., 1989). However, the influence of a 'free' promoter (without the *amdS* gene) on GR efficiency cannot be ruled out.

In conclusion, for the two genes analyzed in this study, it was found that introduction of the gene replacement cassette by *Agrobacterium*-mediated transformation resulted in a higher GR frequency when compared to the $CaCl_2$/PEG protoplast transformation. Furthermore, it seems that shorter or different sized flanks can be used for efficient gene targeting in AMT of *A. awamori* thus, facilitating the generation of gene replacement cassettes by PCR-based methods. Finally, it has been shown that the *amdS* gene can be used as a second selection marker in gene replacement events and that its use leads to a 2-fold enrichment of putatively gene replacement transformants.

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