Transcriptional control of pectin degrading enzymes in Aspergillus niger

Niu, J.; Niu J.

Chapter 2

Efficient Generation of *Aspergillus niger* Knock Out Strains by Combining NHEJ Mutants and a Split Marker Approach

Mark Arentshorst, Jing Niu and Arthur F.J. Ram

Published in *Genetic Transformation Systems in Fungi*, edited by M. A. van Berg and K. Maruthachalam. Volume 1, Fungal Biology

Abstract

To generate gene deletion mutants in *Aspergillus niger*, we combined the use of Non-Homologous End Joining (NHEJ) mutants (*ku70* mutant) and the split marker approach. The combination of both tools resulted in efficient PCR amplification because of the reduced length of the PCR fragments and efficient homologous recombination frequencies. A set of five selection markers, two dominant selection markers (*hph*; hygromycin B resistance and BLE; phleomycin resistance) and three auxotrophic markers (*pyrG*, *argB* and *nicB*) were successfully used in a split marker approach to obtain *amyR* knock outs with high efficiency. *AmyR* encodes a transcription factor that is required for the expression of starch degrading enzymes and disruption of *amyR* results in the inability to grow on starch. The strategy to generate the gene deletion constructs is such that with one set of four gene specific primers, a gene deletion mutant can be generated with either one of the five selection markers. The strategy is based on fusion PCR and omits the necessity for cloning the disruption cassettes. This accelerates the process of generating gene deletion cassettes which can now be accomplished within eight hours. The split marker approach can also be used to make gene deletions in a wild-type background instead of a *Δku70* background. In this chapter, we present protocols and considerations that we used to generate gene knock out constructs by fusion PCR and to obtain and verify gene knock outs with any of the five marker genes using the split marker approach. The method is easily transferable to other filamentous fungi.

Keywords

Aspergillus niger, ku70, split marker approach, five available selection markers.

1. Introduction

Targeted deletion of a Gene of Interest (GOI) is a powerful method to address gene functions and requires a double crossover homologous recombination event to exchange the GOI with a selection marker. In filamentous fungi, DNA integrates preferably via the Non-Homologous End Joining (NHEJ) pathway, which results in low frequencies of homologous recombination and consequently, in low efficiencies in obtaining gene deletion mutants. A successful approach to obtain gene deletion mutants with high efficiency has been the construction of mutants in the NHEJ-pathway, first described for *Neurospora crassa* (Ninomiya *et al.* 2004), and followed up by numerous other filamentous fungi including *Aspergillus niger* (Meyer *et al.* 2007; Carvalho *et al.* 2010; Arentshorst *et al.* 2012). Most often the fungal gene homologous to the gene encoding the Ku70 is used to generate a NHEJ mutant, but also Ku80 and Lig4 homologs have been disrupted to obtain NHEJ-deficient mutants (for reviews see (Meyer 2008), (Kuck and Hoff 2010) and references therein). The use of NHEJ mutants has greatly reduced time and effort to generate gene deletion mutants. The construction of a gene deletion cassette is also an important and time consuming factor. In principle, a gene deletion construct consists of a selection marker, flanked by upstream (5') and downstream (3') sequences of the GOI. Several approaches to generate gene deletion cassettes include traditional restriction enzyme and ligation based cloning, GATEWAY cloning, fusion PCR, or *in vivo* assembly either in *E. coli* or *S. cerevisiae*.

An additional tool for improving gene targeting efficiencies is making use of the split marker technology. In this approach the gene deletion construct is split in two parts and each part contains the flanking region and a truncated form of the selection marker (Fairhead *et al.* 1996; Nielsen *et al.* 2006; Goswami 2012).

For the selection of transformants in *A. niger* (and also other filamentous fungi) the number of available markers is limited. Dominant selection markers for *A. niger* include markers giving resistance to hygromycin (pAN7.1) (Punt *et al.* 1987) or phleomycin (pAN8.1) (Punt and Van Den Hondel 1992), which are well established and commonly used. The uridine and arginine markers (*pyrG* (An12g03570) and *argB* (An14g03400), respectively), have been described earlier and are used in this study (Buxton *et al.* 1985; Van Hartingsveldt *et al.* 1987; Lenouvel *et al.* 2002). The *pyrG* gene encodes for the enzyme orotidine-5'-phosphate-decarboxylase and is required for uracil biosynthesis. The *argB* gene, encoding for an ornithine carbamoyltransferase, is essential for arginine biosynthesis. In addition, a new auxotrophic mutant which requires nicotinamide for growth based on the *nicB* gene (An11g10910) was made. The *A. niger nicB* gene encodes a nicotinate-nucleotide

pyrophosphorylase. Identification and the construction of a gene deletion cassette to disrupt *nicB* is based on a previous work by (Verdoes *et al.* 1994), and will be described elsewhere in detail (Niu et al. manuscript in preparation). The *ΔnicB* strain is auxotrophic for nicotinamide and needs supplementation of nicotinamide to be able to grow. In addition, we reconstructed an *argB* deletion mutant (Niu et al. manuscript in preparation) to have all auxotrophic strains in the same strain background (Table 1).

Table 1. Strains used in this study.

Strain	Genotype	Description	Reference
N402	*cspA1*	derivative of N400	(Bos *et al.* 1988)
AB4.1	*cspA1, pyrG378*	UV mutant of N402	(Van Hartingsveldt *et al.* 1987)
MA169.4	*cspA1, pyrG378, kusA::DR-amdS-DR*	*ku70* deletion in AB4.1	(Carvalho *et al.* 2010)
MA234.1	*cspA1, kusA::DR-amdS-DR*	*ku70* deletion in N402	Arentshorst (unpublished)
JN1.17	*cspA1, pyrG378, kusA::DR-amdS-DR argB::hph*	*argB* deletion in MA169.4	Niu *et al*. unpublished
JN4.2	*cspA1, pyrG378, kusA::DR-amdS-DR nicB::hph*	*nicB* deletion in MA169.4	Niu *et al*. unpublished

Growth of all three auxotrophic strains (*pyrG*$^-$, *argB*$^-$ and *nicB*$^-$) on minimal medium requires the addition of uridine, L-arginine or nicotinamide, respectively[1], and no growth is observed in the absence of the relevant supplements (data not shown). To minimize homologous recombination of the selection markers used in the disruption cassettes, the *argB* and *nicB* homologs from *A. nidulans* (ANID_04409.1 and ANID_03431.1 respectively) and the *pyrG* homolog from *A. oryzae* (AO090011000868) were PCR amplified. All genes are able to complement the auxotrophy of the relevant strain. The hygromycin and phleomycin cassettes also contain only non-homologous sequences as both resistance genes are flanked by the *A. nidulans gpdA* promoter (*PgpdA*) and *trpC* terminator *(TtrpC)* (Table 2).

Table 2. Plasmids to amplify selection markers.

Plasmid	Selection marker	Remark	Reference
pAN7.1	Hygromycin; *hph*	*Pgpd* and *TtrpC* from *A.nidulans*	(Punt *et al.* 1987)
pAN8.1	Phleomycin; BLE	*Pgpd* and *TtrpC* from *A.nidulans*	(Punt and Van Den Hondel 1992)
pAO4-13	*pyrG*	*pyrG* from *A.oryzae*	(De Ruiter-Jacobs *et al.* 1989)
pJN2.1	*argB*	*argB* from *A. nidulans*	Niu *et al*. unpublished
pJN4.1	*nicB*	*nicB* from *A. nidulans*	Niu *et al*. unpublished

[1] The *argB* and *nicB* auxotrofic mutants are also *pyrG*$^-$ and therefore the growth medium for these strains needs to be supplemented with uridine.

2. General Methods

2.1 General split marker approach

The split marker approach used for deleting the GOI is schematically depicted in Figure 1 and consists of two overlapping DNA fragments to disrupt the GOI. The first fragment contains the 5'flank of the GOI and a truncated version of the selection maker. The second DNA fragment contains an overlapping, but truncated version of the selection marker and the 3'flank of the GOI. Both fragments are generated by fusion PCR as described below and transformed simultaneously to the recipient *A. niger* strain. The truncation of the selection marker at either site of the construct results in a non-functional marker and as a consequence transformation of only a single split marker fragment does not result in any transformants (data not shown).

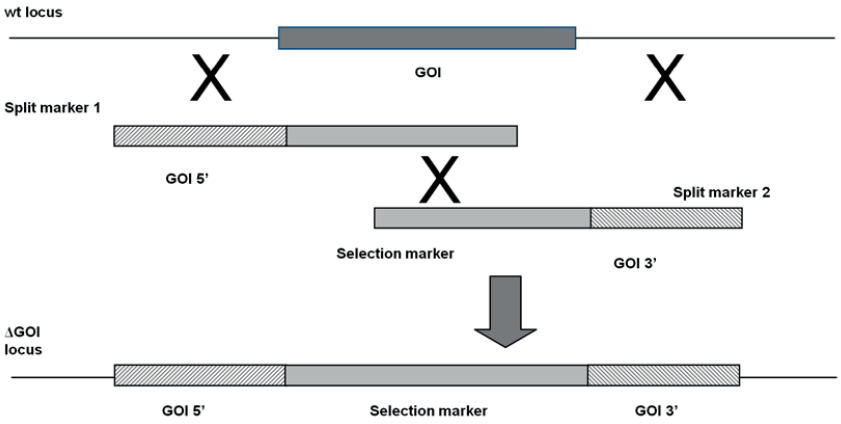

Figure 1. Schematic representation of the split marker gene deletion approach. 5' and 3'seqences flanking the GOI (5'and 3') are transformed simultaneously to the recipient strain. By recombination of the selection maker and homologous integration of the cassette in the genome, a successful gene deletion mutant can be obtained.

2.2 Generation of split marker fragments for *Aspergillus niger* transformation

In this section the experimental design for creating the split marker fragments is discussed. The split marker DNA fragments can be obtained in three steps (Figure 2). Each step is described in detail below.

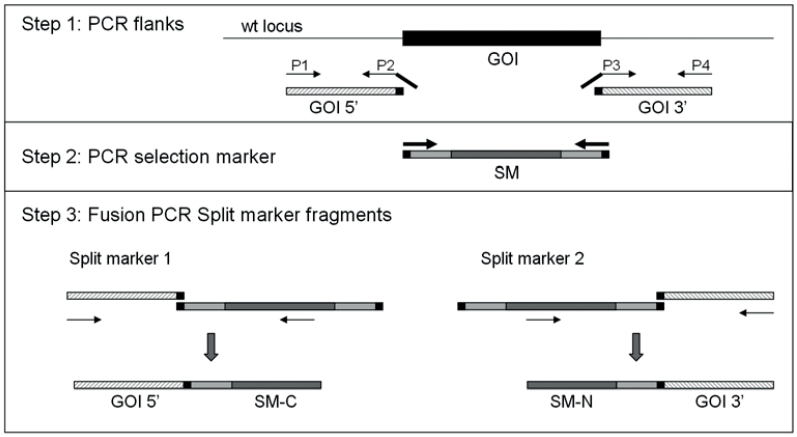

Figure 2. Experimental design for creating split marker fragments.

2.2.1 Experimental design for amplification of flanking regions of the GOI (Step 1)

Once the GOI has been identified, primers need to be designed for making gene deletion cassettes. First, two primers are required for the amplification of the 5' flank of the GOI. The first primer (P1) is chosen between 700 and 900 bases upstream of the start codon. The reverse primer (P2) is as close to the start codon as possible and contains a 5'-CAATTCCAGCAGCGGCTT-3' sequence, which is overlapping with all five selection markers and included for the subsequent fusion PCR. Also, two primers are required for the amplification of the 3' flank of the GOI (P3 and P4). Again, the aim is to generate a 700-900 base pair long flank. In this case, the forward primer (P3) needs a 5'-ACACGGCACAATTATCCATCG-3' sequence, which is also overlapping with all five selection markers for the subsequent fusion PCR (Step3).

2.2.2 Experimental design for amplification of suitable selection marker (Step 2)

For the amplification of the PCR fragments containing the appropriate selection marker the following plasmids can be used (see also Table 2):

The plasmid pAN7.1 (Punt *et al.* 1987) is used as template to amplify the hygromycin resistance cassette, containing the *hph* gene from *E. coli*, coding for hygromycin B phosphotransferase. Expression of the *hph* gene is driven by the *A. nidulans gpdA* promoter, and terminated by the *A. nidulans trpC* terminator. The plasmid pAN8.1 (Punt and Van Den Hondel 1992) is used as template to amplify the phleomycin resistance cassette, containing the BLE gene from *Streptoalloteichus hindustanus*, coding for a phleomycin binding protein.

Expression of the BLE gene is also driven by the *A. nidulans gpdA* promoter and terminated by the *A. nidulans trpC* terminator. The plasmid pAO4-13 (De Ruiter-Jacobs *et al.* 1989) is used as template to amplify the *A. oryzae pyrG* gene (AO090011000868), including promoter and terminator region. The *argB* gene (ANID_04409.1) and the *nicB* gene (ANID_03431.1) of *A. nidulans*, including promoter and terminator region, were amplified using primer pairs argBnidP5f and argBnidP6r or nicBnidP5f and nicBnidP6r, and genomic DNA of *A. nidulans* strain FGSC A234 (*yA2, pabaA1, veA1*), obtained from the Fungal Genetics Stock Center, as template. The resulting PCR products were ligated into PCR-cloning vector pJet1.2 (K1231, Thermo Fisher), to give plasmids pJN2.1 and pJN4.1 respectively (Table 2). Plasmid pJN2.1 and pJN4.1 can be used to amplify the *argB* gene or the *nicB* gene.

We developed a generic split marker approach in such a way that with a single set of four GOI primers, all five different selection markers can be used to generate the deletion cassette. Each primer, used to amplify a specific selection marker (Figure 3, Table 3), contains sequences which are overlapping with the GOI primer sequences (see section 2.2.1) to create gene deletion mutants with either one of the different selection markers.

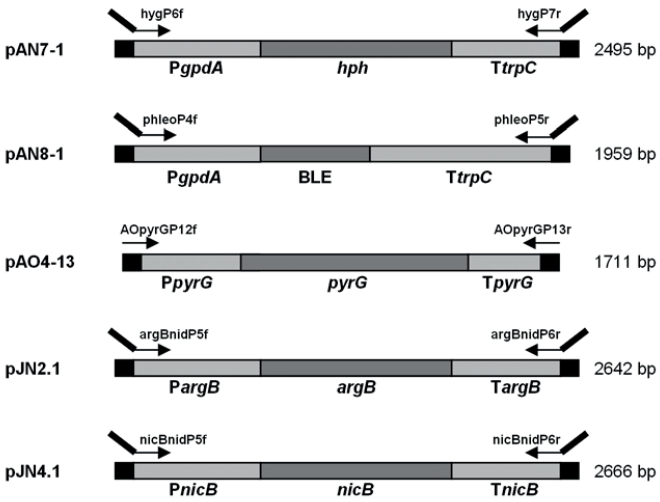

Figure 3. PCR products for all five selection markers. Overlapping sequences of the primers are indicated by bold lines. The size of the PCR products is indicated for each selection marker.

2.2.3 Experimental design for the generation of split marker fragments (Step 3)

Once both flanks of the GOI (Fig. 2, step 1) and the required selection marker (Fig. 2 step 2 and Fig. 3) have been amplified, the split marker fragments can be obtained by fusion PCR (Fig. 2, step 3). Exact details are described in section 3.2.3. After column purification, the resulting split marker fragments can directly be used to transform *A. niger*[2].

3. Detailed procedure description

As proof of principle, the *A. niger amyR* gene (An04g06910), encoding the amylase transcriptional regulator, has been used. The *ΔamyR* strain cannot grow on starch, allowing an easy screen for *ΔamyR* transformants (Petersen *et al.* 1999). This section contains a detailed description of the whole procedure of deleting *amyR*, using all five selection markers, illustrated with results of the experiments. Sequences of all primers used are listed in Tables 3, 4, and 5.

Table 3. Primers used to generate selection markers.

Primer name	Sequence (5'-3')	Remark	Template
hygP6for	**AAGCCGCTGCTGGAATTG-**GGCTCTGAGGTGCAGTGGAT	Amplification of hph marker	pAN7.1
hygP7rev	**CGATGGATAATTGTGCCGTGT-**TGGGTGTTACGGAGCATTCA	Amplification of hph marker	pAN7.1
phleoP4for	**AAGCCGCTGCTGGAATTG -**CTCTTTCTGGCATGCGGAG	Amplification of BLE marker	pAN8.1
phleoP5rev	**CGATGGATAATTGTGCCGTGT-**GGAGCATTCACTAGGCAACCA	Amplification of BLE marker	pAN8.1
AOpyrGP12f	**AAGCCGCTGCTGGAATTG**	Amplification of *pyrG* marker	pAO4-13
AOpyrGP13r	**CGATGGATAATTGTGCCGTGT**	Amplification of *pyrG* marker	pAO4-13
argBnidP5f	**AAGCCGCTGCTGGAATTG -**TTTCGACCTCTTTCCCAATCC	Amplification of *argB* marker	pJN2.1
argBnidP6r	**CGATGGATAATTGTGCCGTGT-**TCCTGTGGGTCTTTGTCCG	Amplification of *argB* marker	pJN2.1
nicBnidP5f	**AAGCCGCTGCTGGAATTG-**CGTTATGCACAGCTCCGTCTT	Amplification of *nicB* marker	pJN4.1
nicBnidP6r	**CGATGGATAATTGTGCCGTGT-**GCGCATACACAGAAGCATTGA	Amplification of *nicB* marker	pJN4.1

Note: overlapping sequences for fusion PCR are indicated in *bold*.

[2] A small sample of PCR fragments is routinely analyzed for purity and size. Optional is to confirm PCR product integrity by restriction analysis or sequencing.

Table 4. GOI (amyR) specific primers to amplify 5'and 3'flanks.

Primer name	Sequence (5'-3')	Remark
amyRP7f	ATCGTCAGCGAGCCTCAGA	Amplification of *amyR* 5'flank
amyRP8r	**CAATTCCAGCAGCGGCTT-**TTGTATGCGGAGACAAGTGTGAC	Amplification of *amyR* 5'flank
amyRP9f	**ACACGGCACAATTATCCATCG-**CCCTCATGAACAAGAAGCAGC	Amplification of *amyR* 3'flank
amyRP10r	GAGGACGCCATCATTGACG	Amplification of *amyR* 3'flank

Note: overlapping sequences for fusion PCR are indicated in *bold*.

Table 5. Generic primers used to amplify bipartite fragments.

Primer name	Sequence (5'-3')	Remark
hygP9r	GGCGTCGGTTTCCACTATC	reverse primer split marker fragment 1 *hph*
hygP8f	AAAGTTCGACAGCGTCTCC	forward primer split marker fragment 2 *hph*
phleoP7r	CACGAAGTGCACGCAGTTG	reverse primer split marker fragment 1 BLE
phleoP6f	AAGTTGACCAGTGCCGTTCC	forward primer split marker fragment 2 BLE
AOpyrGP15r	CCGGTAGCCAAAGATCCCTT	reverse primer split marker fragment 1 *pyrG*
AOpyrGP14f	ATTGACCTACAGCGCACGC	forward primer split marker fragment 2 *pyrG*
argBnidP8r	TGGTTTGCAGAAGCTTTCCT	reverse primer split marker fragment 1 *argB*
argBnidP7f	ACTCCTCGCAAACCATGCC	forward primer split marker fragment 2 *argB*
nicBnidP8r	GAACAGCCTTCGGGATTGC	reverse primer split marker fragment 1 *nicB*
nicBnidP7f	CGCCTTATATCCGATTGGCT	forward primer split marker fragment 2 *nicB*

3.1 Materials and Reagents

For the medium composition of minimal medium, the preparation of stock solutions for the medium and for a detailed protocol of genomic DNA isolation of *A. niger* we refer to the Materials and Reagents section in Arentshorst *et al*. 2012.

1. PCR enzyme (we routinely use Phire Hot start II DNA Polymerase (F-122L, Thermo Fisher).
2. dNTPs (1.25 mM): Add 0.25 mL of all 4 dNTPs (dNTP Set 100 mM Solutions (4 x 0.25 mL, R0181, Thermo Fisher)) to 19 mL of MQ, mix well, make aliquots of 0.5 mL and store at -20˚C.
3. PCR purification Kit (we routinely use Genejet Gel Extraction Kit (K0692, Thermo Fisher), also for PCR purifications).
4. Hygromycin (100 mg/mL): Dissolve 1 g of hygromycin (InvivoGen, ant-hg-10p) in 10 mL of MQ, sterilize by filtration, make aliquots of 500 µL and store at −20°C. The final concentration in the medium is 100 µg/mL, except for transformation plates, then use 200 µg/mL.
5. Phleomycin (40 mg/mL), for 10 mL: add 400 mg of phleomycin (InvivoGen, ant-ph-10p) to 8 mL of warm MQ (~60˚C) in a 15 mL tube. When phleomycin is dissolved, add MQ up to 10 mL and filter sterilize. Make aliquots and store at -20˚C.

6. Uridine (1 M), for 100 mL: add 22.4 g of uridine (Acros, 140775000) to 50 mL of warm MQ (~60˚C) in a 100 mL cylinder. When uridine is dissolved, add MQ up to 100 mL, sterilize by filtration and store at 4°C. Final concentration in medium is 10 mM.
7. Arginine (2%), for 100 mL: add 2 g of L-arginine monohydrochloride (Sigma, A5131) to 50 mL of warm MQ (~60˚C) in a 100 mL cylinder. When arginine is dissolved, add MQ up to 100 mL, sterilize by filtration and store at 4˚C.
8. Nicotinamide (0.5%), for 100 mL: add 0.5 g of nicotinamide (Sigma, N0636) to 50 mL of warm MQ (~60˚C) in a 100 mL cylinder. When nicotinamide is dissolved, add MQ up to 100 mL, sterilize by filtration and store at 4˚C.
9. Transformation media + phleomycin: Prepare MMS and Top agar according to Arentshorst et al. 2012. After autoclaving, and cooling down to 50ºC, add phleomycin to a final concentration of 50 µg/mL, to both the MMS and the Top agar.
10. MM + agar + L-arginine: Prepare 500 mL of MM + agar according to Arentshorst et al. 2012. Add 5 mL of 2% L-arginine after autoclaving (100 x dilution).
11. MM + agar + nicotinamide: Prepare 500 mL of MM + agar according to Arentshorst et al. 2012. Add 0.25 mL of 0.5% nicotinamide after autoclaving (2000 x dilution).
12. MM + agar + starch: For 500 mL: Dissolve 5 g of starch (soluble, extra pure, Merck, 1.01253) in 450 mL of warm MQ (~60˚C). Add 10 mL of 50 x ASP+N, 1 ml of 1M $MgSO_4$, 50 mL of trace element solution, 15 mg of yeast extract (YE)[3] (Roth, 2363.2) and 7.5 g agar bact. (Scharlau, 07-004-500), and autoclave.

3.2 Methods

*3.2.1 Amplification of the amyR 5'- and 3' flan*k

1. *AmyR* primers were designed (Fig. 2, Step 1 and Table 4), and subsequently used in PCR reactions to amplify both the *amyR* 5' flank and 3' flank.
2. The PCR mix, total volume of 50 µL, contained 1 µL genomic DNA of *A. niger* wt strain N402 (1µg/µL), 8 µL dNTP's (1.25 mM), 10 µL 5x Phire buffer, 1 µL Primer F (20 pmol/µL), 1 µL Primer R (20 pmol/µL), 0.5 µL Phire Hot start II DNA Polymerase and 28.5 µL of MQ.

[3] YE is added to a final concentration of 0.003% to stimulate germination of *A.niger*. On MM + starch without YE, the wt strain also does not grow very well.

3. PCR was performed under the following conditions: initial denaturation for 5 min at 98˚C, 30 cycles of 5 sec at 98˚C, 5 sec at 58˚C, and 15 sec per 1 kb of template at 72˚C, followed by final extension of 5 min at 72˚C.
4. PCR reactions were analyzed by loading 5 µL PCR reaction on a 1% agarose gel.
5. After column purification and elution with 30 µL of MQ, DNA concentration for both flanks was ~37 ng/µL.

3.2.2 Amplification of the selection markers

1. Primers for all five selection markers were designed (Fig 3, Table 3) and used for PCR. In these PCR reactions 1 ng of plasmid (pAO4-13, pAN7.1, pAN8.1, pJN2.1 and pJN4.1, respectively) was used as template. For PCR mix and PCR conditions see section 3.2.1.
2. After confirmation on agarose gel, selection marker PCR products were column purified, yielding DNA concentrations of ~50 ng/µL. The markers were stored at -20˚C and used repeatedly.

3.2.3 Amplification of the split marker fragments

1. Fusion PCR fragments were amplified according to Figure 2, step 3 (see also Table 5 and 6). Both *amyR* flanks and all selection markers (section 3.2.1 and 3.2.2) were diluted to 2 ng/µL.
2. For each PCR reaction, 2 ng of *amyR* flank and 2 ng of selection marker PCR were used as template (Table 6). For PCR mix and PCR conditions see section 3.2.1.
3. Two identical fusion PCR reactions were performed, in order to increase the yield of PCR product.
4. Fusion PCR products were analyzed on agarose gel, followed by column purification. The DNA concentration for all fragments varied between 120-160 ng/µL in a total volume of 20 µL (Table 6, column DNA Yield)[4].

[4] The split marker fragments are not purified from gel and template DNA (pyrG, hygB, Ble , argB , and nicB genes, respectively) used for amplification of the split marker might remain present in the next steps. We therefore include control transformations with both split markers separately. As no transformants are obtained in the transformation with only one flank (data not shown), the purification of the split marker fragment is not required, but is optional.

Table 6. Overview of templates and primers used in Fusion PCR reactions to obtain split marker fragments.

Split Marker 1					Split Marker 2					Transformed
Template		Primer		Product	Template		Primer		Product	Strain
1	2	Forward	Reverse	Yield (µg)	1	2	Forward	Reverse	Yield (µg)	
5'amyR	PCR hph	amyRP7f	hygP9r	2.5	3'amyR	PCR hph	hygP8f	amyRP10r	2.6	MA234.1
5'amyR	PCR BLE	amyRP7f	phleoP7r	2.6	3'amyR	PCR BLE	phleoP6f	amyRP10r	2.6	MA234.1
5'amyR	PCR pyrG	amyRP7f	AOpyrG15r	2.8	3'amyR	PCR pyrG	AOpyrGP14f	amyRP10r	2.8	MA169.4
5'amyR	PCR argB	amyRP7f	argBnidP8r	3.0	3'amyR	PCR argB	argBnidP7f	amyRP10r	3.2	JN1.17
5'amyR	PCR nicB	amyRP7f	nicBnidP8r	3.3	3'amyR	PCR nicB	nicBnidP7f	amyRP10r	2.5	JN4.2

3.2.4 Transformation of split marker fragments to Aspergillus niger Δku70 strains

1. Split marker fragments were combined and transformed to different *A. niger* strains (Table 6, column Transformed strain), according to Arentshorst *et al*. 2012. Results of these transformations are shown in Figure 4.
2. As a control, also separate split marker fragments were transformed. None of the separately transformed split marker fragments yielded any transformants (data not shown).
3. Four transformants were purified for each selection marker tested[5]. For purification protocol, see Arentshorst *et al*. 2012.
4. After the second purification, all purified transformants were tested for growth on MM + starch (Fig.4). All transformants analyzed showed a *ΔamyR* phenotype.
5. Purified transformants can be further analyzed by isolating genomic DNA, followed by both Southern blot analysis and diagnostic PCR (Arentshorst *et al*. 2012).

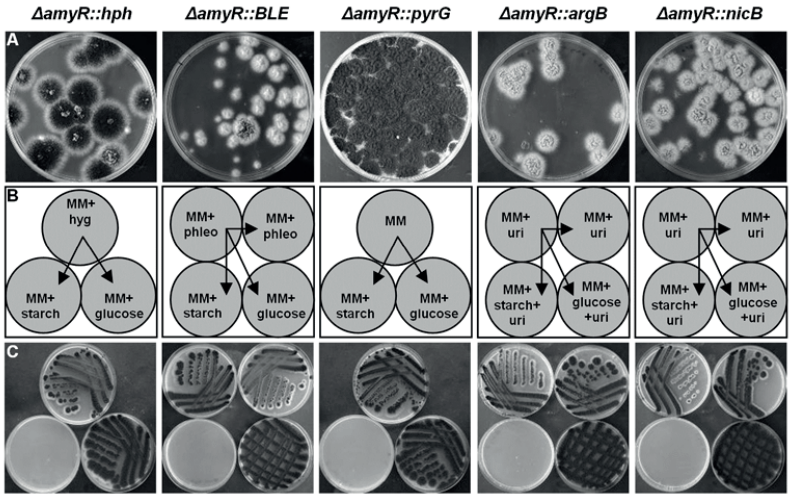

Figure 4. Phenotypic analysis of putative *amyR* disruptant strains using five different selection markers (*hph* = hygromycin resistance; BLE = phleomycin resistance; *pyrG* = uridine requiring; *argB* = arginine requiring; *nicB* = nicotinamide requiring). A) Transformation plates after transforming split marker fragment combinations for each of the five *amyR* deletion cassettes to the relevant recipient strain (Table 6). B, C) Purified transformants were analysed for their ability to grow on starch. The inability to grow on starch is indicative for the deletion of the *amyR* gene.

[5] Only the sporulating transformants on the phleomycin transformation plate (see Fig. 4) can grow on MM + phleomycin. The non-sporulating transformants do not grow, and are probably transient transformants, in which the split marker fragments have not integrated into the genome.

3.2.5 Transformation of split marker fragments to Aspergillus niger wt strains

For some experimental set-ups, it is preferred to analyze gene deletions in a *ku70* wild type strain. In order to show that the split marker approach also can be applied to a wild type (*ku70* plus) strain, both *A. niger* strains AB4.1 (Van Hartingsveldt *et al.* 1987) (*pyrG*$^{-}$) and MA169.4 (*Δku70, pyrG*$^{-}$) were transformed with *ΔamyR::pyrG* split marker fragments. After purification and screening on MM + starch, 25 out of 60 AB4.1-transformants (41%) showed a *ΔamyR* phenotype[6]. For MA169.4, 39 out of 40 transformants (98%) showed a *ΔamyR* phenotype. This result clearly shows that the split marker approach can also be used to make gene deletions in a wt background instead of a *Δku70* background.

[6] The percentages of Homologous Recombination (HR) for the *amyR* gene are very high (41% for wt, 98% for *Δku70*). Usually we find 5-10% HR for wt, and 80-100% for *Δku70Meyer, V., M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra et al., 2007 Highly efficient gene targeting in the Aspergillus niger kusA mutant. J Biotechnol 128: 770-775, ibid. ibid.*(Meyer et al. 2007).

Acknowledgments

Jing Niu was supported by a grant from the China Scholarship Council. The research group of A.F.J. Ram is part of the Kluyver Centre for Genomics of Industrial Fermentation which is supported by the Netherlands Genomics Initiative.

References

Arentshorst, M., A. F. Ram and V. Meyer, 2012 Using non-homologous end-joining-deficient strains for functional gene analyses in filamentous fungi. Methods Mol Biol 835**:** 133-150.

Bos, C. J., A. J. Debets, K. Swart, A. Huybers, G. Kobus *et al.*, 1988 Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr Genet 14**:** 437-443.

Buxton, F. P., D. I. Gwynne and R. W. Davies, 1985 Transformation of *Aspergillus niger* using the argB gene of *Aspergillus nidulans*. Gene 37**:** 207-214.

Carvalho, N. D., M. Arentshorst, M. Jin Kwon, V. Meyer and A. F. Ram, 2010 Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl Microbiol Biotechnol 87**:** 1463-1473.

de Ruiter-Jacobs, Y. M., M. Broekhuijsen, S. E. Unkles, E. I. Campbell, J. R. Kinghorn *et al.*, 1989 A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. Curr Genet 16**:** 159-163.

Fairhead, C., B. Llorente, F. Denis, M. Soler and B. Dujon, 1996 New vectors for combinatorial deletions in yeast chromosomes and for gap-repair cloning using 'split-marker' recombination. Yeast 12**:** 1439-1457.

Goswami, R. S., 2012 Targeted gene replacement in fungi using a split-marker approach. Methods Mol Biol 835**:** 255-269.

Kuck, U., and B. Hoff, 2010 New tools for the genetic manipulation of filamentous fungi. Appl Microbiol Biotechnol 86**:** 51-62.

Lenouvel, F., P. J. van de Vondervoort and J. Visser, 2002 Disruption of the *Aspergillus niger* argB gene: a tool for transformation. Curr Genet 41**:** 425-432.

Meyer, V., 2008 Genetic engineering of filamentous fungi--progress, obstacles and future trends. Biotechnol Adv 26**:** 177-185.

Meyer, V., M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra *et al.*, 2007 Highly efficient gene targeting in the *Aspergillus niger* kusA mutant. J Biotechnol 128**:** 770-775.

Nielsen, M. L., L. Albertsen, G. Lettier, J. B. Nielsen and U. H. Mortensen, 2006 Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. Fungal Genet Biol 43**:** 54-64.

Ninomiya, Y., K. Suzuki, C. Ishii and H. Inoue, 2004 Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci U S A 101**:** 12248-12253.

Petersen, K. L., J. Lehmbeck and T. Christensen, 1999 A new transcriptional activator for amylase genes in *Aspergillus*. Mol Gen Genet 262**:** 668-676.

Punt, P. J., R. P. Oliver, M. A. Dingemanse, P. H. Pouwels and C. A. van den Hondel, 1987 Transformation of Aspergillus based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56**:** 117-124.

Punt, P. J., and C. A. van den Hondel, 1992 Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol 216**:** 447-457.

van Hartingsveldt, W., I. E. Mattern, C. M. van Zeijl, P. H. Pouwels and C. A. van den Hondel, 1987 Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet 206**:** 71-75.

Verdoes, J. C., P. J. Punt, P. van der Berg, F. Debets, A. H. Stouthamer *et al.*, 1994 Characterization of an efficient gene cloning strategy for *Aspergillus niger* based on an autonomously replicating plasmid: cloning of the *nicB* gene of *A. niger*. Gene 146**:** 159-165.