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Expanding the *ku70* toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses

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Abstract Mutants with a defective non-homologous-end-joining (NHEJ) pathway have boosted functional genomics in filamentous fungi as they are very efficient recipient strains for gene-targeting approaches, achieving homologous recombination frequencies up to 100%. For example, deletion of the *ku70* homologous gene *kusA* in *Aspergillus niger* resulted in a recipient strain in which deletions of essential or non-essential genes can efficiently be obtained. To verify that the mutant phenotype observed is the result of a gene deletion, a complementation approach has to be performed. Here, an intact copy of the gene is transformed back to the mutant, where it should integrate ectopically into the genome. However, ectopic complementation is difficult in NHEJ-deficient strains, and the gene will preferably integrate via homologous recombination at its endogenous locus. To circumvent that problem, we have constructed autonomously replicating vectors useful for many filamentous fungi which contain either the *pyrG* allele or a hygromycin resistance gene as selectable markers. Under selective conditions, the plasmids are maintained, allowing complementation analyses; once the selective pressure is removed, the plasmid becomes lost and the mutant phenotype prevails. Another disadvantage of NHEJ-defective strains is their increased sensitivity towards DNA damaging conditions such as radiation. Thus, mutant analyses in these genetic backgrounds are limited and can even be obscured by pleiotropic effects. The use of sexual crossings for the restoration of the NHEJ pathway is, however, impossible in imperfect filamentous fungi such as *A. niger*. We have therefore established a transiently disrupted *kusA* strain as recipient strain for gene-targeting approaches.

Keywords *Aspergillus niger* · *ku70* · AMA1 · Heterokaryon rescue · Transformation

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Introduction

Integration of DNA sequences into a genome by homologous recombination is a very useful and widely used functional genomics tool for generating gene knock-out mutants. In filamentous fungi such as *Aspergillus niger*, homologous recombination frequencies are extremely low when compared to the yeast *Saccharomyces cerevisiae,* which makes the generation of homologous transformants time-consuming. When Ninomiya et al. (2004) reported that inactivation of components of the non-homologous-end-joining (NHEJ) pathway in *Neurospora crassa* results in strains with homologous recombination frequencies up to 100%, this system was rapidly established in other filamentous fungi (for reviews see (Meyer 2008, Kück and Hoff 2010)). The NHEJ pathway is a conserved mechanism in eukaryotes that is essential for the repair of chromosomal DNA double-strand breaks (DSBs) and competes with another conserved repair mechanism, the homologous recombination (HR) pathway (Shrivastav et al. 2008). The HR pathway depends on the Rad52 epistasis group and mediates interaction between homologous DNA sequences leading to targeted integration. In contrast, the

NHEJ pathway ligates DSBs without the requirement of any homology and is accomplished by the activities of the Ku heterodimer (Ku70/Ku80–protein complex) and the DNA ligase IV–Xrcc4 complex (Dudasova et al. 2004; Krogh and Symington 2004). By deleting either *ku70*, *ku80* or *lig4* genes, non-homologous recombination is diminished or prevented, favouring the frequencies of homologous recombination events mediated by the Rad52 complex (Shrivastav et al. 2008).

Despite the fact that NHEJ-defective mutants are powerful recipient strains for fungal gene-targeting approaches, a problem arises when one wants to complement the phenotype of a gene deletion mutant. Usually, this is performed by retransferring the respective gene back into the deletion mutant. The gene will ectopically integrate into the genome and the resulting phenotype is assessed. However, in a NHEJ-deficient background, ectopic integration is not favoured and the complementing gene construct will preferably integrate at its endogenous (deleted) locus. One possibility to bypass this disadvantage is the use of autonomously replicating vectors, where the complementing gene construct is maintained extrachromosomally. For *Aspergilli*, the use of the AMA1 sequence has been shown to promote extrachromosomal replication of a plasmid. The autonomous maintenance in *Aspergillus* (AMA1) sequence, isolated from a genomic library of *Aspergillus nidulans*, is based on two inverted sequences surrounding a unique central core sequence and displays properties similar to the autonomous replicating sequences in *S. cerevisiae* (Gems et al. 1991; Verdoes et al. 1994; Aleksenko and Clutterbuck 1996, 1997; Khalaj et al. 2007).

We thus established in this work AMA1-based complementing vectors conferring either uracil prototrophy (*pyrG*) or hygromycin resistance for general use in filamentous fungi. In addition, we generated a set of isogenic Δ*ku70* deletions strains for *A. niger*, which can be used for a variety of selection markers. To test the usefulness of the AMA1-based complementation approach, two genes (*hacA* and *ireA*) have been selected which are linked to our research interest on secretion-related phenomena in *A. niger*. HacA is a transcription factor important for the unfolded protein response (UPR) and activates transcription of various chaperones and foldases (Mulder et al. 2004; Mulder et al. 2006). IreA is the predicted homologue of the *S. cerevisiae* Ire1p, which is a conserved transmembrane protein located in the endoplasmic reticulum (ER) membrane (Cox et al. 1993). ER stress in yeast and mammals is sensed by Ire1p which in turn stimulates the Hac1p-UPR machinery (Patil and Walter 2001). We report here that deletion of *hacA* or *ireA* causes dramatic consequences for *A. niger* and demonstrate that the AMA1-based plasmids are exceptional useful tools for complementation analyses of essential genes.

A second drawback of NHEJ inactivation is that the consequences of deleting *ku70*, *ku80* or *lig4* in relation to DNA repair and genome stability are less studied in fungal strains. However, as several reports have shown that NHEJ deficiency makes fungal strains vulnerable to DNA damaging conditions (Malik et al. 2006; Meyer et al. 2007; Kito et al. 2008; Snoek et al. 2009), it can be assumed that an intact NHEJ pathway secures cellular fitness of filamentous fungi as shown for higher eukaryotes (Pardo et al. 2009). To avoid any limitations provoked by a non-functional NHEJ pathway, Nielsen et al. (2008) used a strategy to transiently silence the NHEJ pathway in *A. nidulans*. We have adapted that strategy to *A. niger* and report here the establishment of a transiently disrupted *ku70* (*kusA*) strain. This strain shows similar homologous recombination frequencies compared to a *ΔkusA* strain as exemplarily shown for two genes of our interest—*srgA* and *racA*. Both are GTPase-encoding genes and are important for secretion and morphology of *A. niger* ((Punt et al. 2001) and own unpublished data).

Materials and methods

Strains, culture conditions and molecular techniques

A. niger strains used in this study are listed in Table 1. Strains were cultivated in minimal medium (MM; (Bennett and Lasure 1991)) containing 55 mM glucose, 7 mM KCl, 11 mM KH_2PO_4, 70 mM $NaNO_3$, 2 mM $MgSO_4$, 76 nM $ZnSO_4$, 178 nM H_3BO_3, 25 nM $MnCl_2$, 18 nM $FeSO_4$, 7.1 nM $CoCl_2$, 6.4 nM $CuSO_4$, 6.2 nM Na_2MoO_4, 174 nM EDTA; or in complete medium containing, in addition to MM, 0.1% (*w*/*v*) casamino acids and 0.5% (*w*/*v*) yeast extract. When required, 10 mM uridine and/or 100 μg/ml of hygromycin was added. When using the *amdS* as selection marker, strains were grown in MM without $NaNO_3$ and supplemented with 10 mM acetamide and 15 mM cesium chloride.

To obtain *pyrG*$^-$ strains, 2×10^7 spores were inoculated on MM agar plates supplemented with 0.75 mg/ml 5′-fluoroorotic acid (FOA), 10 mM uridine and 10 mM proline as nitrogen source. Plates were incubated for 1–2 weeks at 30°C. FOA-resistant mutants were isolated, purified and tested for uridine auxotrophy on MM with and without uridine (mutants should not grow on medium lacking uridine). To obtain *amdS*$^-$ strains, 2×10^7 spores were inoculated on MM agar plates supplemented with 0.2% 5′-fluoroacetamide (FAA) and 10 mM urea as nitrogen source. After 1–2 weeks incubation at 30°C, FAA-resistant mutants were isolated, purified and tested for growth on acetamide medium (mutants should not grow on medium containing acetamide as sole nitrogen source).

Table 1 *Aspergillus niger* strains used in this study

Name	Genotype	Reference
N402	*cspA1, amdS*$^-$	Bos et al. (1988)
AB4.1	*pyrG*$^-$, *amdS*$^-$	van Hartingsveldt et al. (1987)
MA70.15[a]	*ΔkusA, pyrG*$^-$, *amdS*$^+$	Meyer et al. (2007)
MA78.6[a]	*ΔkusA, amdS*$^+$	This study
NC4.1[a]	*ΔkusA, pyrG*$^-$, *amdS*$^-$	This study
NC5.1[a]	*ΔkusA, pyrG*$^+$, *amdS*$^-$	This study
NC6.2	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔhacA*	This study
NC7.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔireA/ireA*	This study
NC8.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔhacA*, pAMA-hacA	This study
NC9.1	*ΔkusA, pyrG*$^+$, *amdS*$^+$, *ΔireA,* pAMA-ireA	This study
MA169.4[a]	*kusA*::DR-*amdS*-DR, *pyrG*$^-$	This study
MA171.1	*kusA*::DR-*amdS*-DR, *pyrG*$^+$, *ΔracA*	This study
MA172.1	*kusA*$^+$, *pyrG*$^+$, *ΔracA*	This study
MK15.A	*Δkus*, *pyrG*$^+$, *ΔsrgA*	This study
MK18.A	*kusA*::DR-*amdS*-DR, *pyrG*$^+$, *ΔsrgA*	This study

[a] Strains have been deposited at the Fungal Genetics Stock Center (www.fgsc.net)

All basic molecular techniques were performed according to standard procedures (Sambrook and Russel 2001). Transformation of *A. niger*, genomic DNA extraction, screening procedures, diagnostic PCR and Southern analysis were conducted as recently described in detail (Meyer et al. 2010).

Construction of AMA1-based complementation vectors

The pBlueScriptII SK (Stratagene) was used as a backbone for the construction of the autonomously replicating plasmids. The *hph* expression cassette was obtained by digesting pAN7.1 (Punt et al. 1987) with *Xho*I and *Hin*dIII. The *AopyrG* gene was obtained by PCR using pAO4-13 (de Ruiter-Jacobs et al. 1989) as template DNA and primers pAO-XhoI-Rev and pAO-HindIII-For which introduced the restrictions sites *Xho*I and *Hin*dIII, respectively, into the *AopyrG* fragment (Table 2). The 3-kb *hph* cassette and the 1.7-kb *AopyrG* fragment were independently cloned into *Xho*I/*Hin*dIII-digested pBlueScriptII SK, giving rise to vectors pBS-hyg and pBS-pyrG, respectively. The 6-kb AMA1 fragment was obtained by digesting pAOpyrGcosArp1 (Gems et al. 1991) with *Hin*dIII. The fragment was then cloned into the unique *Hin*dIII site in pBS-hyg and pBS-pyrG vectors, giving plasmids pBS-hyg-AMA (pMA171) and pBS-pyrG-AMA (pMA172), respectively.

Generation of Δ*hacA* and Δ*ireA* deletion strains

The *A. niger hacA* gene (An01g00160) was deleted in MA70.15 by replacing its open reading frame (ORF) with a DNA fragment containing the *pyrG* marker form *Aspergillus oryzae* (van Hartingsveldt et al. 1987). The cassette used for *hacA* deletion was produced by fusion-PCR in two steps: first, independent amplification of the *hacA* promoter and terminator regions (each≅550 bp) and the *AopyrG* gene, respectively, using primers summarised in Table 2. Genomic DNA of strain N402 and pAB4-1 (van Hartingsveldt et al. 1987) served as template DNA. Second, fusion-PCR using the three fragments as template DNAs and NC16hacA5F/NC19hacA3R as outward primers (Table 2).

Deletion of the *A. niger ireA* ORF (An01g06550) followed the same approach as described for *hacA*. Respective primers are listed in Table 2. The deletion cassettes were transformed into MA70.15 and uridine prototrophic transformants were selected and analysed by Southern hybridisation.

Analysis of Δ*hacA* and *ΔireA* deletion strains

Spores from primary transformants were carefully removed to prevent transfer of mycelia and conidiophores using a sterile cotton stick moistened in 0.9% NaCl and suspended in 10 ml 0.9% NaCl. Spores were plated out on MM (selective) and MM+uridine (non-selective) agar plates and incubated at 30°C for 5 days. In the case that an essential gene has been deleted, no colonies will be formed under selective conditions, as both the deletion strain and the parental strain are not able to grow. Thus, such heterokaryons can only be propagated by transferring mycelium. The heterokaryon rescue technique (Osmani et al. 2006) was used to purify the poor-growing *hacA* deletion mutant. No viable *ireA* deletion mutants could be obtained after purification of the primary transformants containing the *ireA* deletion. Propagation and maintenance of *ireA* heterokaryotic strains was done by transfer of mycelia from the primary transformants onto MM. Putative *ΔhacA* and *ΔireA* heterokaryotic mutants were further analysed by Southern hybridisation.

Table 2 Primers used in this study

Primer name	Sequence (5′ to 3′)	Targeted sequence
pAO-HindIII-For	CCCAAGCTTGTTGCTCGGTAGCTGATTA	*AopyrG*
pAO-XhoI-Rev	CCGCTCGAGCGATGGATAATTGTGCCG	*AopyrG*
NC14 pyrGfor	GGATCTCAGAACAATATACCAG	*AopyrG*
NC15 pyrGrev	CCGCTGTCGGATCAGGATTA	*AopyrG*
NC16 hacA5F	CATATTCACCCAACCGGACG	*hacA* 5′deletion flank
NC17 hacA5R	CTGGTATATTGTTCTGAGATCCAACAATGGCAACTCAGGCGT	*hacA* 5′deletion flank
NC18 hacA3F	TAATCCTGATCCGACAGCGGTCGCCAGACTTCTAGCGTGC	*hacA* 3′deletion flank
NC19 hacA3R	GGTAGTAAAGTCTCACCGCTG	*hacA* 3′deletion flank
NC20 ireA 5F	CGTCCGTGCATCTGGCTTA	*ireA* 5′deletion flank
NC21ireA5R	CTGGTATATTGTTCTGAGATCCCCTTCGCTGATCGCTGTCTCT	*ireA* 5′deletion flank
NC22 ireA 3F	TAATCCTGATCCGACAGCGGTGAGCTGCCACTCCGTCAT	*ireA* 3′deletion flank
NC23 ireA3R	GGGGATTGGTGTACTTACGG	*ireA* 3′deletion flank
NC58 hacA R	ATAAGAATGCGGCCGCCATCCCGATTGCCGTATCC	*hacA* ORF
NC8 PHACF2	ATAAGAATGCGGCCGCCTCCATACCACTTTGTGCTAG	*hacA* ORF
NC60 ireA R	ATAAGAATGCGGCCGCGGGGATTGGTGTACTTACGG	*ireA* ORF
NC61 ireA F	ATAAGAATGCGGCCGCCGTCCGTGCATCTGGCTTA	*ireA* ORF
P1—NC46 5′ku70 F	GGTGCGAGAAGCCGGTCGCA	*kusA*—diagnostic PCR
P2—NC48 amdS Rev	AGAGAGGACGTTGGCGATTG	*kusA*—diagnostic PCR
P3—NC47 3′ ku70 R	TTACGGCGAATCTGGGTGG	*kusA*—diagnostic PCR
P4—NC49 ku70 orf R	AAATGAGTGCGACGCGGA	*kusA*—diagnostic PCR
ku70P1Not	AAGGAAAAAAGCGGCCGCCAGAACGGCTTGATGACGG	*kusA* 5′disruption flank
ku70P2EcoRI	CACAGAATTCGACCTCATGAGCCGAAGGAA	*kusA* 5′disruption flank
ku70P3Kpn	GGGGTACCACCAGTCAAAGATGCGGTCC	*kusA* 3′disruption flank
ku70P4Kpn	GGGGTACCGGCGCTTGCCTTCGTAAGA	*kusA* 3′disruption flank

Restriction sites added are underlined

For complementation studies using pMA171, the ORFs of *hacA* and *ireA*, including approximately 0.6 kb promoter and 0.6 kb terminator regions, were PCR-amplified using N402 genomic DNA as template and respective primers containing *Not*I overhangs (Table 2). The fragments were cloned into pJET (Fermentas), sequenced, released from pJET via *Not*I restriction and cloned into *Not*I-linearised pMA171. Respective plasmids (pMA171-hacA and pMA171-ireA) were then transformed into the *hacA* and *ireA* deletion mutants. Primary transformants containing the complementation plasmid were isolated on MM containing 100 μg/ml of hygromycin and further analysed by Southern blot. To provoke plasmid loss, spores were streaked for several rounds on non-selective medium (MM without hygromycin).

Generation of isogenic *kusA* deletion strains

A *kusA* deletion construct, consisting of the *amdS* selection marker flanked by each 1.5 kb of 5′ and 3′ regions of *kusA* and localised on plasmid pGBKUS-5 (Meyer et al. 2007) was used to transform the *A. niger* wild-type strain N402 (Bos et al. 1988). Transformants in which the *kusA* gene was replaced by *amdS* were selected on acetamide agar plates and via Southern blot analysis as described (Meyer et al. 2007). The resulting strain MA78.6 (*ΔkusA*, *amdS*+, $pyrG^{+}$) was used for further studies. In order to loop-out the *amdS* marker, strains MA78.6 and MA70.15 (*ΔkusA*, *amdS*+, $pyrG^{-}$, (Meyer et al. 2007)) were plated on MM agar plates containing FAA. FAA-resistant strains were selected, subjected to Southern analysis and analysed by diagnostic PCR using primers P1–P4 (Table 2). Strains NC4.1 (*ΔkusA*, $amdS^{-}$, $pyrG^{-}$) and NC5.1 (*ΔkusA*, $amdS^{-}$, $pyrG^{+}$) were selected.

Generation of a *kusA* disruption strain and restoration of the *kusA* locus

A *kusA* disruption construct (*kusA*::DR-*amdS*-DR) was made based on the *kusA* deletion construct pGBKUS-5 (Meyer et al. 2007). PCR 1 using primers ku70P1Not and ku70P2Eco was used to amplify a 700-bp region covering part of the 5′ untranslated region of *kusA* and its ORF. The primer pair ku70P3Kpn and ku70P4Kpn was used in PCR

2 to amplify a 900-bp region from the *kusA* ORF. Both PCR products contained the same 300 bp sequence from the *kusA* ORF (later on named direct repeat, DR). Using a two-step ligation, PCR product 1 (restricted with *Not*I and *Eco*RI), the *amdS* gene cassette (released via *Eco*RI and *Kpn*I restriction from pGBKUS-5) and PCR product 2 (restricted with *Kpn*I) were cloned into pBlueScriptII SK giving plasmid pMA183. The *kusA*::DR-*amdS*-DR cassette was amplified from pMA183 using primers ku70P1Not and ku70P4Kpn and transformed into strain AB4.1 (van Hartingsveldt et al. 1987). Transformants, in which the *kusA* locus was disrupted by the DR-flanked *amdS* marker gene, were screened for growth on acetamide and via Southern blot analysis. Strain MA169.4 was selected (*kusA*::DR-*amdS*-DR) and subsequently used as recipient strain for deleting *srgA* and *racA*. The *A. niger srgA* gene (An14g00010) was deleted in MA169.4 using the 5.3-kb *Eco*RI–*Bam*HI fragment from plasmid pΔsrgA as described earlier (Punt et al. 2001). The deletion construct contained the *Trichoderma reesei pyr4* gene as selection marker (Gruber et al. 1990). The gene deletion approach followed for *racA* will be described elsewhere (Kwon, Meyer, Ram et al; manuscript in preparation).

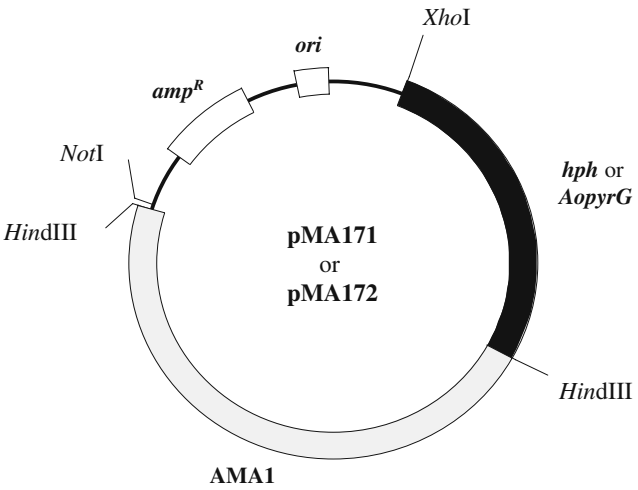

Fig. 1 Schematic representation of the autonomously replicating plasmids containing either the hygromycin resistance gene *hph* (pMA171; 11,239 bp) or *AopyrG* (pAM172; 9,943 bp) as selection markers. Sites for restriction enzymes used for cloning are indicated. Both plasmids have been deposited at FGSC. *hph* hygromycin resistance gene from *E. coli* under control of the *gpdA* promoter and the *trpC* terminator of *A. nidulans*, *AopyrG A. oryzae pyrG* gene flanked by its own promoter and terminator sequences, *ori* origin of replication in *E. coli*, amp^R gene conferring ampicillin resistance in *E. coli*

Results

Establishment of AMA1-based vectors as molecular tools for complementation analyses

We have constructed autonomously replicating vectors containing either the auxotrophic selection marker *pyrG* of *A. oryzae* encoding an orotidine-5′-monophosphate decarboxylase or harbouring the hygromycin resistance cassette as dominant selection marker. Both markers have already successfully been used for a variety of filamentous fungi. A schematic drawing of both plasmids pMA171 (hygromycin-based) and pMA172 (*AopyrG*-based) is given in Fig. 1. Common to both shuttle vectors is the pBluescript backbone and the 6-kb AMA1 sequence, allowing autonomous maintenance in *Escherichia coli* and filamentous fungi. We further ensured that a unique rare-cutting restriction site is present in both plasmids (*Not*I) which should facilitate easy insertion of complementing genes.

Analysis of *hacA* and *ireA* deletion strains and heterokaryon rescue

In order to judge the usefulness of the AMA1-based complementation tool, we deleted two genes of our research interest on protein secretion in *A. niger*—*hacA* and *ireA*. In doing so, the uridine-requiring strain MA70.15 (*ΔkusA, pyrG*$^-$, *amdS*$^+$) was selected as a recipient strain (Fig. 2d). The respective deletion cassettes were made using the *AopyrG* gene as a selection marker (for details see "Materials and methods"). After transforming strain MA70.15 with the deletion cassettes, no obvious phenotypes were observed for the primary transformants obtained in both gene deletion approaches (data not shown), and each four primary transformants were randomly selected for purification. Hereby, only conidiospores from the primary transformants were transferred onto new selective medium (MM without uridine; note that conidia of *A. niger* are uninucleate). Remarkably, the phenotype of the four putative *ΔhacA* transformants did no longer resemble the wild-type's phenotype, but instead all strains displayed reduced growth and formed compact colonies (Fig. 2b). In the case of *ΔireA* transformants, none of the four primary transformants formed colonies after transfer (Fig. 2a). These results indicated that the primary transformants of both deletion approaches were heterokaryons, containing nuclei with the genotype *hacA/pyrG*$^-$ (*ireA/pyrG*$^-$) and nuclei with the genotype *ΔhacA/pyrG*$^+$ (*ΔireA/pyrG*$^+$). We were only able to obtain pure, homokaryotic transformants in the case of the *ΔhacA* transformants (compact growing colonies), whereas propagation of the *ΔireA* transformants was only possible when substrate mycelium was transferred (data not shown). This finding suggested that *ΔireA* strains are only viable as heterokaryons and that *ireA* is an essential gene. To confirm the homokaryotic genotype of the purified *ΔhacA* transformants, mycelium from a *ΔhacA* colony were transferred on MM plates containing uridine. If still wild-type nuclei

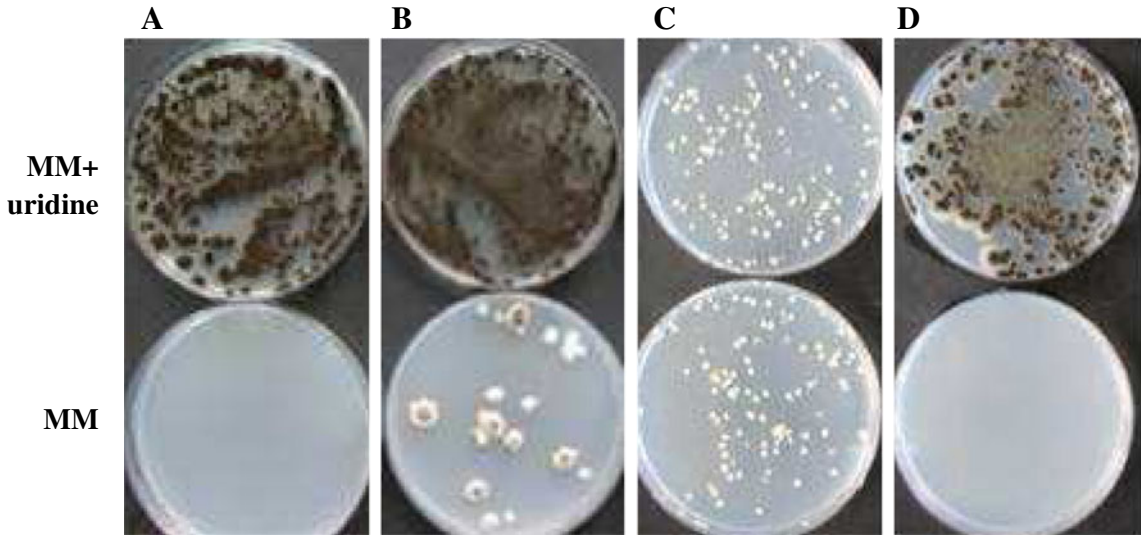

Fig. 2 Heterokaryon tests of primary transformants from putative *ireA* (**a**) and *hacA* (**b**) deletion strains on MM agar plates supplemented or lacking uridine. To prove purity of the *ΔhacA* strain, spores from the putative deletion strain were grown in the presence of uridine (**c**). As a control, growth of the parental strain MA70.15 (*ΔkusA, pyrG*$^{-}$*, amdS*$^{+}$) is shown (**d**)

would have been present (*hacA/pyrG*$^{-}$), vigorous growth would have been observable on MM+uridine plates. As shown in Fig. 2c, the phenotype of the *ΔhacA* transformant is stable on MM+uridine plates, indicating the absence of wild-type nuclei and proves that the strain is homokaryotic.

In order to verify deletion of both genes on molecular level, Southern analyses were conducted. Due to reduced sporulation of the putative *hacA* deletion strain and the heterokaryotic nature of the putative *ΔireA* primary transformants, the isolation of genomic DNA was done by inoculating pieces of mycelium in MM lacking uridine. Southern blot analysis was performed on one *ΔhacA* mutant (NC6.2) and two *ΔireA* primary transformants (NC7.1 and NC7.2) and confirmed deletion of both genes (Fig. 3). For strains NC7.1 and NC7.2, it also confirmed their heterokaryotic nature (signals corresponding to both the presence and absence of the *ireA* allele were observed) and suggested an unbalanced proportion of both nuclei (Fig. 3b, lanes 1 and 2).

Complementation of *ΔhacA* and *ΔireA* with AMA1-based vectors

As the *hacA* and *ireA* deletion strains were established by using *AopyrG* as a selection marker, the AMA1-based vector pMA171 conferring hygromycin resistance was used for complementation experiments. Two vectors, pMA171-hacA and pMA171-ireA, were constructed as described under "Materials and methods" and transformed into a NC6.2 (*ΔhacA*) and NC7.1 (*ΔireA*/*ireA*), giving strains NC8 and NC9, respectively. Transformants were selected and purified on MM lacking uridine but containing hygromycin. Complementing strains were analysed by Southern hybridisation which confirmed, in the case of *ΔhacA*, the presence of the disrupted gene as well as the complementing plasmid (Fig. 3a, lanes 1 and 2). For the

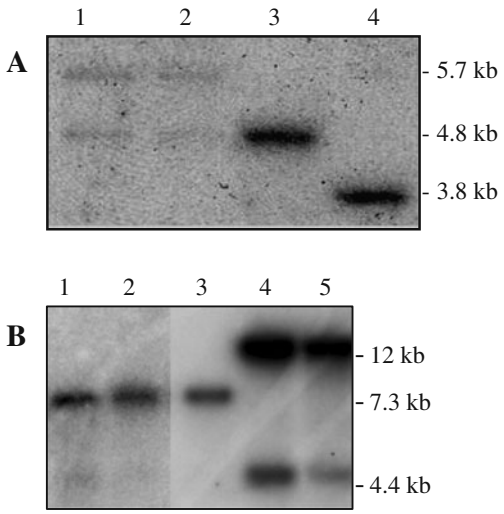

Fig. 3 Southern analysis of *ΔhacA* (**a**) and *ΔireA* (**b**) strains. **a** Genomic DNA was digested with *Eco*RI and probed with a 560-bp fragment corresponding to the *hacA* 3′-untranslated region. *Lanes 1* and *2*: two strains (NC8.1 and NC8.2) derived from NC6.2 transformed with pMA171-hacA; *lane 3*: NC6.2 (*ΔhacA*); *lane 4*: N402 (*hacA*). Predicted sizes of the hybridising fragments are as predicted and indicated on the *left*. **b** Genomic DNA was digested with *Kpn*I and probed with a 600-bp fragment of *ireA* 5′-untranslated region. *Lanes 1* and *2*: heterokaryotic NC7.1 and NC7.2 strains (*ΔireA/ireA*). The two signals correspond to the wild-type locus (7.3 kb) and the *ireA* deleted locus (4.4 kb); *lane 3*: N402 (*ireA*); *lanes 4* and *5*: NC9.1 and NC9.2 correspond to two different *ΔireA*-complemented strains derived from NC7.1 transformed with pAM171-ireA. The two signals correspond to the deleted locus (4.4 kb) and the vector (12 kb)

ΔireA-complemented strains, we observed a band pattern corresponding to the presence of the AMA1-plasmid and a deleted *ireA* locus (Fig. 3b, lanes 4 and 5). The difference in band intensities between the complementing plasmid and *ireA* deletion could suggest that more than one plasmid copy is present per nucleus as previously proposed for *A. niger* (Verdoes et al. 1994).

All NC8 transformants obtained grew like the wild-type, indicating that a plasmid-based *hacA* gene can fully restore the severe growth defect provoked by a *hacA* deletion (Fig. 4). In the case of NC9 strains, the lethal *ireA* deletion phenotype was almost fully rescued by a plasmid-based *ireA* gene (Fig. 4). However, the wild-type phenotype was not completely restored, suggesting that the cellular amount of IreA is strongly controlled. Any overexpression, as might have resulted from plasmid-based expression, could have caused a mild stress phenotype.

Interestingly, our results also suggested that the AMA1-based complementation plasmids are rather stably maintained in *A. niger*, as under non-selective conditions they did not easily become lost. However, after multiple rounds of cultivation under non-selective conditions, we observed that the wild-type phenotype (provided by the presence of pMA171 or pMA172) reverted back into the mutant phenotype, i.e., the plasmids became lost (data not shown).

Construction of a set of isogenic *ΔkusA* strains for *A. niger*

In order to expand the repertory of *ΔkusA* strains for *A. niger* offering different choices of selection markers, we established two new isogenic *ΔkusA* strains. The deletion of *A. niger kusA* gene in strain AB4.1 (*pyrG*$^-$, *amdS*$^-$) has previously been reported (Meyer et al. 2007), using a construct consisting of 1.5 kb of 5′- and 3′-flanking regions of the *kusA*, the *amdS* selection marker and a repeat of the 5′ flanking region to facilitate *amdS* removal by recombination. The resulting strain was named MA70.15 (*ΔkusA*, *amdS*$^+$, *pyrG*$^-$; (Meyer et al. 2007)). The same deletion construct was used in the present work to transform the *A. niger* wild-type strain N402 (*amdS*$^-$). Transformants with a deleted *kusA* gene were identified via Southern blot analysis (data not shown), and MA78.6 (*ΔkusA, amdS*$^+$) was selected for further studies. This strain and MA70.15 were subjected to counter-selection using the antimetabolite 5-fluoroacetamide (FAA). The direct repeat of the 5′-flanking region of the *kusA* gene flanking the *amdS* marker allowed efficient loop-out of the *amdS* marker. The correct loop-out of the *amdS* cassette was confirmed by Southern blot (data not shown), and two strains were used for further analysis: NC4.1 (MA70.15 derivative) and NC5.1 (MA78.6 derivative). For rapid strain identification, a diagnostic PCR

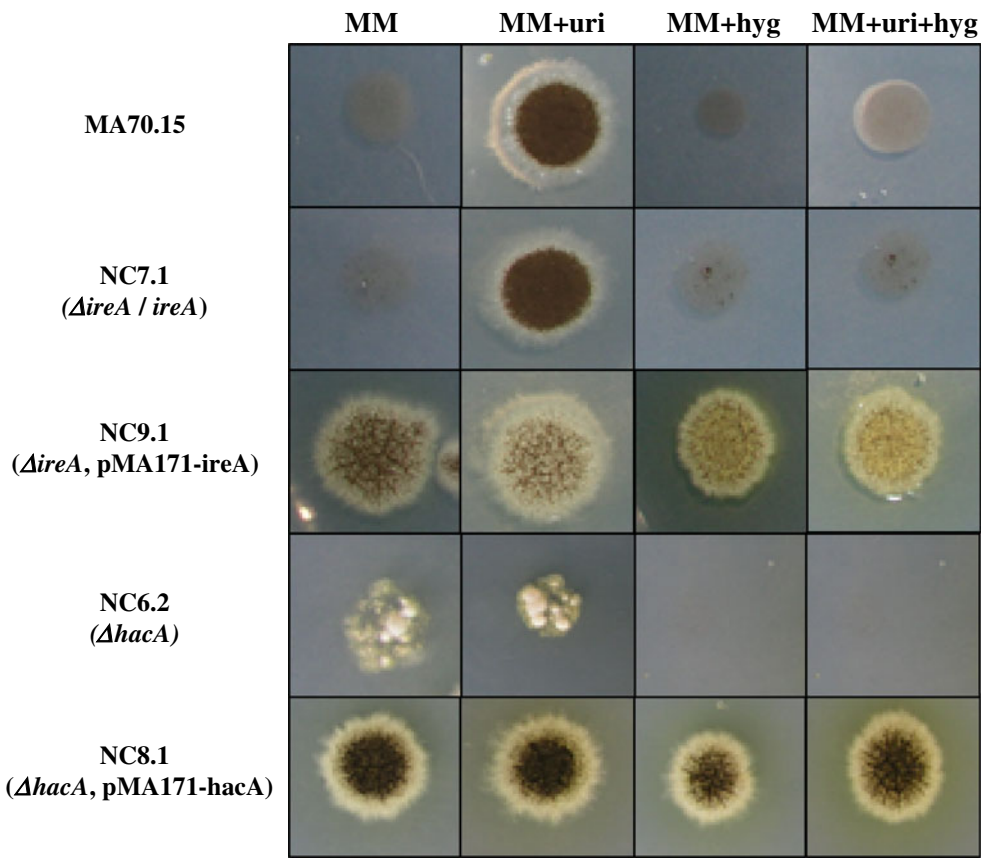

Fig. 4 Phenotypic analysis of *ΔhacA* mutants and *ΔireA/ireA* heterokaryons and respective complemented strains. Spores (10^4) were spotted on the different types of media indicated and incubated at 30°C for 3 days, except for the *ΔhacA* mutant which was cultivated for 6 days. Note that the presence of 100 μg/ml hygromycin allows a slight background growth of *A. niger* (e.g. as seen for MA70.15). To fully repress any growth, 150 μg/ml hygromycin are preferable. *uri* uridine, *hyg* hygromycin

approach was designed to detect the presence and/or absence of *kusA* and *amdS*, respectively. By performing three different PCR reactions with selected combinations of four primers, strains can be identified carrying the wild-type *kusA* locus, the *ΔkusA*(*kusA*::*amdS*) locus or the *ΔkusA* locus, where *amdS* has been looped out (Fig. 5). As described previously for the *ΔkusA* mutant MA70.15 (Meyer et al. 2007), no obvious differences among AB4.1, MA78.6, NC4.1 and NC5.1 were observed with respect to growth, morphology and biomass accumulation (data not shown).

Design and performance of a transient *kusA* disruption strain

To establish a transiently disrupted *kusA* allele in *A. niger*, we adapted a strategy, followed recently for *A. nidulans* (Nielsen et al. 2008). As described in detail in Fig. 6 and in the "Materials and methods" section, a construct was made in which the *amdS* marker is flanked by *kusA* sequences which have in common a direct repeat of 300 bp (DR) from the *kusA* ORF. That construct was transformed into *A. niger* strain AB4.1 (*pyrG*$^-$), and one strain was isolated (out of ten analysed) that carried a disrupted allele of *kusA* (Figs. 6, 7 and data not shown). The strain selected, MA169.4 (*kusA*$^-$, *pyrG*$^-$, *amdS*$^+$), as well as MA70.15 (*ΔkusA, pyrG*$^-$, *amdS*$^+$) were subsequently used as recipient strains for targeted deletion of a GTPase-encoding gene *srgA*. Its deletion phenotype is easy to score because *srgA* null strains display clear defects in growth and are hyper-branching (Punt et al. 2001). About 60 transformants were obtained for each of the transformations, ~95% of which clearly showed the deletion phenotype (data not shown). Hence, both recipient strains ensure similar HR frequencies. A similar conclusion we could draw after performing a gene deletion approach targeting another GTPase-encoding gene, *racA*. Here, about ~55% of the transformants showed the deletion phenotype in both the *kusA* deletion and *kusA* disruption background strains (n>90; data not shown). Three of the *ΔracA* strains (MA171.1–MA171.3), where deletion of *racA* was verified by Southern hybridisation (Fig. 7 and data not shown), were selected and subjected to FAA counter-selection. From the three MA171 strains, each two *amdS*$^-$ colonies were randomly selected (MA172.1–MA172.6) and their *kusA* locus analysed by Southern hybridisation (Fig. 7 and data not shown), PCR-amplified and sequenced. No deviations from the *kusA* wild-type sequence were encountered in all six sequencing reactions (data not shown), demonstrating that the *kusA* gene can accurately and fully be restored by looping out the *amdS* marker via the 300-bp DRs (Fig. 6).

Discussion

The inactivation of the NHEJ pathway has been demonstrated to be a successful tool to perform targeted genetic manipulations in a very efficient manner and has paved the way for high-throughput functional genomics approaches in filamentous fungi. For example, the *A. niger kusA* deletion mutant MA70.15 has been proven to be a powerful recipient strain to generate gene deletions and to identify essential genes (Meyer et al. 2007). To broaden the choice of selection markers for gene-targeting approaches and to avoid uridine/uracil auxotrophic strains when using dominant selection markers (MA70.15 is *pyrG*$^-$), we deleted in

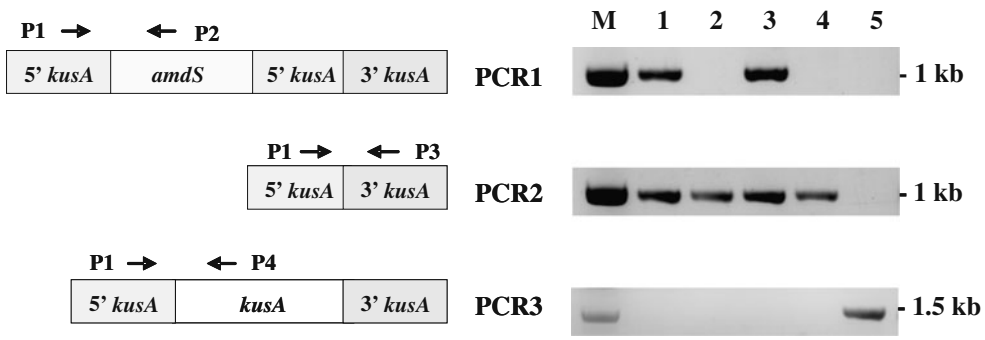

Fig. 5 Diagnostic PCR approach to analyse different genetic backgrounds with respect to the *kusA* locus. Three PCR reactions using different primer combinations were performed using genomic DNA obtained from the following strains (relevant genotypes are given in *brackets*): *lane 1*, MA70.15 (*ΔkusA, amdS*$^+$); *lane 2*, NC4.1 (*ΔkusA, amdS*$^-$); *lane 3*, MA78.6 (*ΔkusA, amdS*$^+$), *lane 4*, NC5.1 (*ΔkusA, amdS*$^-$); *lane 5*, N402 (*amdS*$^-$). In PCR1, only those strains which harbour the *amdS* gene are expected to generate a PCR product (primer combination P1 and P2). In PCR2, a 1 kb amplicon is expected only for *ΔkusA* strains. Under the conditions used (primer combination P1 and P3, short elongation time), the generation of the 1-kb amplicon is favoured over the generation of a 3-kb fragment which would result in MA70.15 and MA78.6 (contain the *amdS* gene between the 5′-*kusA* and 3′-*kusA* flanking regions) and N402 (contains the *kusA* gene between the 5′-*kusA* and 3′-*kusA* flanking regions). In the case of PCR3 (primer combination P1 and P4), only N402 bearing an intact *kusA* locus is expected to generate a 1.5 kb band. The predicted sizes of PCR fragment are indicated. *M* molecular weight marker

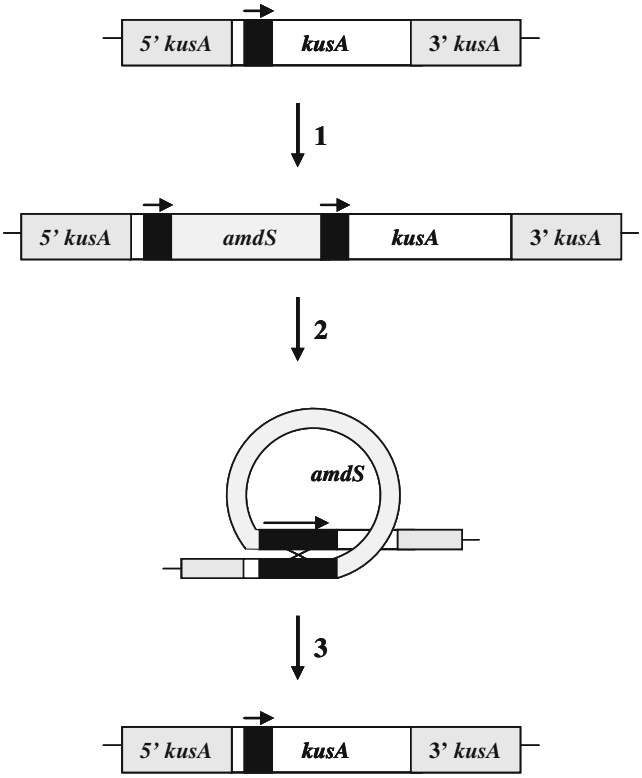

Fig. 6 Strategy for transient disruption of *kusA*. The counter-selectable marker *amdS*, flanked on each site with a 300-bp sequence of the *kusA* ORF (DR, marked by a *black box* and an *arrow*) was integrated into the *kusA* gene (*1*). In the resulting strain MA169.4, carrying the disrupted *kusA* allele, any gene-targeting approach can be performed with high efficiency. After counter-selection on FAA medium (*2*), the *amdS* gene loops out via single crossover between the DR (*3*), and a functional *kusA* allele becomes restored

this study the *kusA* gene in the prototrophic *A. niger* strain N402. The resulting strain MA78.6 has repeatedly been used in our lab for gene deletion approaches using the hygromycin cassette and showed similar HR frequencies as reported for strain MA70.15 (own unpublished results). In addition, both MA70.15 and MA78.6 have been cured for the *amdS* marker by FAA counter-selection, generating NC4.1 (Δ*kusA*, *pyrG*$^{-}$) and NC5.1 (Δ*kusA*), respectively. The now available strain collection of MA70.15, MA78.6, NC4.1 and NC5.1 allows the use of several well-established selection markers (*pyrG*, acetamidase, hygromycin, phleomycin), thus improving flexibility in making user-defined mutations. Furthermore, the generation of prototrophic strains in which at least four genes can be targeted without the need for curing any selection marker has now become feasible.

To further demonstrate the value of Δ*kusA* strains, we have exemplarily focused on deleting two UPR genes, namely *hacA* and *ireA*. The generation of Δ*hacA* and Δ*ireA*/*ireA* strains unambiguously illustrates the advantage of using a NHEJ-deficient strain as recipient for the isolation of mutants displaying a severe growth defect (Δ*hacA)* or for the deletion of essential genes (*ireA*). Our findings that all of the randomly selected primary transformants of Δ*hacA* and Δ*ireA* were heterokaryons, support earlier observations that heterokaryon formation is improved in a Δ*kusA* background strain, probably as a result of less-favoured ectopic integration events (Meyer et al. 2007). The elegant heterokaryon rescue technique useful for studying gene functions in heterokaryons has been first described in *A. nidulans* (Osmani et al. 2006). This technique is especially valuable for the asexual fungus *A. niger*, as it is impossible to generate deletions in a diploid strain and to analyse its progeny after meiosis. A heterokaryon strain can be used instead, and functional analysis tests can be performed by transferring spores or pieces of heterokaryotic mycelium onto different growth plates ((Osmani et al. 2006; Todd et al. 2007) and this work).

Interestingly, the number of nuclei carrying the deleted gene and nuclei carrying the wild-type gene are unbalanced in the Δ*ireA*/*ireA* strains (Fig. 3b). Such a nucleus imbalance is not unusual in fungal heterokaryotic strains and has been reported, e.g. for *A. nidulans*, *A. niger* and *Neurospora crassa* (Punt et al. 1998; Pitchaimani and Maheshwari 2003; Ichinomiya et al. 2007; Todd et al. 2007). As shown for *N. crassa*, the proportion of nuclei depends on medium conditions, the number of sub-cultivations and the genetic locus affected (Pitchaimani and Maheshwari 2003). Unfortunately, such an imbalance limits the use of heterokaryons for haploinsufficiency screens and analyses, which are outstanding tools to study gene functions as shown for *S. cerevisiae* and *Candida albicans* (Giaever et al. 1999; Baetz et al. 2004; Martinez and Ljungdahl 2004). It is conceivable,

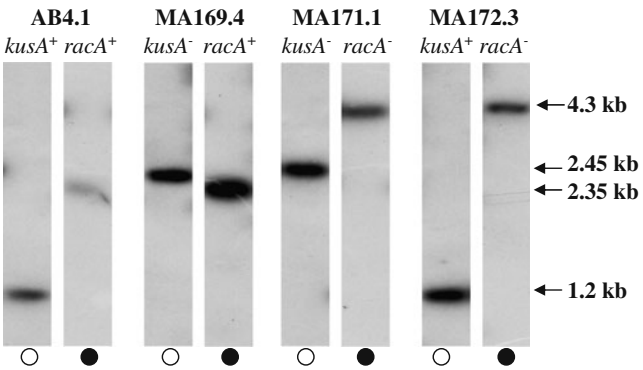

Fig. 7 Southern analysis for strains in which *kusA* has transiently been disrupted. Genomic DNAs of the four strains indicated were restricted with *Nco*I (marked with *open circle*) or *Bgl*II (marked with *closed circle*). *Nco*I restricted DNAs were hybridised with a *kusA* probe targeting the 5′ untranslated region of *kusA*. Those strains which harbour an intact *kusA* allele show a signal at 1.2 kb, whereas strains in which *kusA* has been disrupted with the *amdS* marker display a 2.45 kb signal. *Bgl*II-restricted genomic DNAs were hybridised with a *racA* probe targeting the 5′ untranslated region of *racA*. In case of an intact *racA* allele, a 2.35 kb band will be visible, in case *racA* has been deleted with the *AopyrG* gene, a 4.3 kb band becomes apparent

however, that a balance of both types of nuclei is re-adjustable in heterokaryons, e.g. by fine-tuning the selection pressure, an assumption that awaits experimental verification.

Another drawback of imperfect fungi such as *A. niger* is the circumstance that a defective NHEJ pathway cannot be restored by sexual crossing. However, the NHEJ pathway and its components are crucial for maintaining genome integrity in eukaryotes, especially in aging cells. DSBs can arise from intracellular reactive oxygen species, by replication folk collapse, during meiotic chromosome segregation or from exogenous attacks such as radiation and hazardous chemicals. Consequently, DSB repair mechanisms such as HR and NHEJ pathways are crucial to the survival of eukaryotes (Pardo et al. 2009). To avoid any detrimental and pleiotropic effects of a constantly inactive NHEJ pathway in *A. niger*, we also established strain MA169.4 harbouring a transiently disrupted *kusA* allele. Using two genes of our interest as an example (*srgA*, *racA*), we could show that MA169.4 is as efficient as MA70.15 with respect to introducing gene deletions. The advantage of MA169.4 over MA70.15 is, however, that the native *kusA* allele can easily be restored after the genetic engineering approach has been accomplished. The respective strains gained are then especially valuable when post-exponential or aging phenomena are in the focus of the research. In this context, it is worth mentioning that HR frequencies are not only dependent on the activity of HR and NHEJ, but are also strongly dependent on the gene locus. We have encountered that about 10% of the *A. niger* genes analysed so far in our group (>60 genes), are difficult to target—potentially because they are localised close to contig borders or within silenced heterochromatic DNA regions.

Complementation of gene deletion mutants with the wild-type gene copy is an essential control to prove the function of a gene of interest. There are different options to perform complementation analyses, such as ectopic or homologous integration of the gene copy. Both strategies have their disadvantages, e.g. ectopic integration is difficult to perform in a NHEJ-deficient background, and homologous integration raises questions on the choice of the target locus as it has to ensure sufficient expression of the wild-type gene. In addition, we have encountered that many *A. niger* mutants are difficult to transform if genomic integration has been approached (e.g. *ΔhacA*, data not shown). The AMA1-based complementation strategy described here in this work appeared to be very straightforward and successful to complement gene deletions in *A. niger* (Fig. 4). Moreover, it has also some advantages over the other two options: (1) it is possible to transform AMA1-based plasmids at high frequencies; (2) transformation efficiencies are independent of the *kusA* background; (3) high transformation efficiencies can even be obtained for mutants which are difficult to transform and (4) the selection marker as well as the complementing gene can easily be removed, simply by growing the complemented strains for several generations on non-selective medium ((Gems et al. 1991; Gems and Clutterbuck 1993) and this work).

Taken together, this study has expanded the *ku70* toolbox for *A. niger* by generating various recipient strains for flexible and improved functional gene analysis. In addition, the establishment of the AMA1-based plasmids pMA171 and pMA172 are not only new and valuable molecular tools for *A. niger*, they can moreover be generally implemented for usage in filamentous fungi as the individual gene cassettes present in both plasmids are functional in many Asco- and Basidiomycetes.

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