Reversible impairment of the *ku80* gene by a recyclable marker in *Aspergillus aculeatus*

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**Abstract**

Auxotrophic mutants of *Aspergillus* can be isolated in the presence of counter-selective compounds, but the process is laborious. We developed a method to enable reversible impairment of the *ku80* gene (*Aaku80*) in the imperfect fungus *Aspergillus aculeatus*. *Aaku80* was replaced with a selection marker, orotidine 5'-phosphate decarboxylase (*pyrG*), followed by excision of *pyrG* between direct repeats (DR) to yield the *Aaku80* deletion mutant (MR12). The gene-targeting efficiency at the ornithine carbamoyltransferase (*argB*) locus was drastically elevated from 3% to 96% in MR12. The frequency of marker recycling depended on DR length. One uridine auxotroph was obtained from 3.3 × 10⁵, 1.4 × 10⁵, and 9.2 × 10³ conidia from strains harboring 20-, 98-, and 495-bp DRs, respectively. Because these strains maintained the short DRs after 5 d of cultivation, we investigated whether *Aaku80* function was disrupted by *pyrG* insertion with the 20-bp DR and restored after excision of *pyrG*. The *Aaku80* disruption mutant (coku80) was bred by inserting *pyrG* sandwiched between 20-bp DRs into the second intron of *Aaku80*, followed by excision of *pyrG* between the DRs to yield the coku80rec strain. Analyses of homologous recombination frequency and methyl methanesulfonate sensitivity demonstrated that *Aaku80* function was disrupted in coku80 but restored in coku80rec. Furthermore, *pyrG* was maintained in coku80 at least for ten generations. These data indicated that reversible impairment of *ku80* in *A. aculeatus* is useful for functional genomics in cases where genetic segregation is not feasible.

**Keywords:** Aspergillus aculeatus, Homologous recombination, Ku gene, Counter selection, Marker recycling

**Introduction**

*Aspergillus aculeatus* [NBRC 108796], a filamentous fungus isolated from the soil, produces a number of extracellular cellulose- and hemicellulose-degrading enzymes that exhibit synergism with *Trichoderma* cellulases. Alkali-treated rice straw was shown to be hydrolyzed almost completely into monosaccharides by mixed-culture filtrate of these two species (Murao et al. 1979, 1988). Previously, we constructed a host–vector system of *A. aculeatus* that expressed the *A. aculeatus* β-mannosidase gene (Kanamasa et al. 2003). The *A. aculeatus* expression system yielded a 10-fold higher level of the enzyme than did an *Aspergillus oryzae* expression system, indicating the high potential of *A. aculeatus* as a factory to produce useful proteins (Kanamasa et al. 2007). However, the concerted action of several cellulases, each with different properties, is considered to be essential for the effective hydrolysis of cellulose into monosaccharides. To breed *A. aculeatus* that simultaneously produces many types of cellulases, a marker-recycling technique, enabling to excise a selection marker gene from transformants without leaving exogenous DNA fragments, must be developed (Akada et al. 2006). This technique enables the excision of a selection marker gene from transformants without leaving exogenous DNA fragments; multiple cellulase genes can be introduced into a host strain with a limited number of selection markers. Furthermore, elucidating how the expressions of the cellulose-degrading enzyme-encoding genes are regulated will improve enzyme production. Therefore, one must develop highly-efficient homologous recombination (HR) techniques as well as a marker-recycling technique in the imperfect fungus *A. aculeatus*.

Recently, deletion of the *ku70*/*80* or *lig4* genes, which are both involved in DNA double strand repair, was demonstrated to elevate the frequency of HR in filamentous fungi (Ishibashi et al. 2006; Krappmann et al. 2006;
Mizutani et al. 2008; Ninomiya et al. 2004; Takahashi et al. 2006). These achievements enabled the study of the cellular roles of interesting genes. Based on this information, we cloned and replaced the A. *aculeatus* *ku80* gene with a selection marker to yield the A. *aculeatus* *ku80* deletion mutant. We also investigated the length of the direct repeats (DRs) required for efficient marker recycling in the A. *aculeatus* *ku80* deletion mutant to enable a reversirepulsion of *ku80*.

Materials and methods

Strains

All A. *aculeatus* strains used in this study were derived from wild-type A. *aculeatus* no. F-50. Unless otherwise stated, all strains were propagated at 30°C in appropriately supplemented minimal medium (MM) (Adachi et al. 2009). The *pyrG* (orotidine 5'-phosphate decarboxylase) gene-deficient mutants (uridine auxotrophs) were spontaneously isolated by spreading 1 × 10⁶ A. *aculeatus* wild-type conidia on a plate containing 1 mM 5-fluoroorotic acid (5-FOA) and 0.4 mM uridine. Colonies that showed a 5-FOA-resistant phenotype were isolated and transformed with the *Aspergillus nidulans* *pyrG* gene (*ApyrG*), and strains that showed uridine prototrophy were regarded as *pyrG* deficient mutants (pyrG13, uridine auxotrophs), which were used as hosts to breed arginine prototrophs and the deletion mutant in isolated by spreading 1 × 10⁶ deficient mutants (uridine auxotrophs) were spontaneously shown uridine prototrophy were regarded as A. *nidulans* pyrG.

Supplemented minimal medium (MM) (Adachi et al. 2009).

Nucleic acid analysis

PCR was performed using PrimeSTAR HS DNA polymerase (Takara, Kyoto, Japan) as described in the manufacturer’s instructions, except that the annealing temperature was 55°C and there were 30 PCR cycles. All primers used in this study are shown in Table 1. Genomic DNA was isolated from ground mycelia with Micro Smash MS-100R (Tomy, Tokyo, Japan) as described previously (Adachi et al. 2009). Southern hybridization was performed as described previously (Adachi et al. 2009).

Cloning the A. *aculeatus* *ku80* and ornithine carbamoyltransferase genes

A part of the A. *aculeatus* *ku80* gene (Aaku80) was amplified from the A. *aculeatus* genome by PCR using the primers ku80F1 and ku80R3, which were designed to anneal to the region conserved among the *Aspergillus* *ku80* genes. The 1.2-kb PCR product was inserted into the EcoRV site of pBluescript II KS (+) (pBS II KS (+)) (Stratagene, La Jolla, CA, USA), and both strands were sequenced twice. Southern blot analysis of the *Aaku80* gene with a selection marker suggested that a 6-kb genomic DNA fragment digested with *Sphi*I possessed the Aaku80 gene. This fragment was then cloned into the *Sphi*I site of pUC118 (Takara) to yield pAaku80.

Table 1 List of primers used in this study

| Primers used to clone the Aaku80 segment |
|----------------------------------------|
| ku80F1 | 5'-ATGGCNGNAAGGAAGCAACNGT-3' |
| ku80R3 | 5'-CGTCCATAYTCATANCYTYTGCC-3' |
| Primers used to clone the Aaarg8 segment |
| AaargBF3 | 5'-CARGCNATHGNCAYTTYAIRC-3' |
| AaargBR2 | 5'-GGNARRCARTGCAATRAAYTTCCA-3' |
| Primers used for the Aaku80 deletion |
| aku80AF1 | 5'-GCCGCCGCGATGTTCTATGTGCTTGCC-3' |
| aku80AR2 | 5'-CGACTGGGTCTCTCCTGCGATGCGATGCCGAAG-3' |
| aku80BF1 | 5'-TGGTATTGACGCACTAGTATGATGGCACAAGCAG-3' |
| aku80BR1 | 5'-GCCGCCGCCATTGAGTCTGGGTGAC-3' |
| aku80CF1 | 5'-CTTCTGTGCTACTCGCCATGCCGACAGAGACATTCCG-3' |
| aku80CR1 | 5'-GAGACATTGTCTGGCACTTGAGTGGGTTG-3' |
| npyrF2 | 5'-CAACCCCACTCCACCATCCCGCAGACATCTCTC-3' |
| npyrR1 | 5'-CTCGGTGGACACATCATGCTGGCTGCAATACCA-3' |
| Primers used for the A. *nidulans* pyrF insertion into Aaku80 |
| coku80NF | 5'-TAGATATACGTCCTTCTGACCCATGC-3' |
| coku80NR | 5'-TTACGGCAGCGGTAGAAACCATCATGCTTGCTGAC-3' |
| coku80CF | 5'-GCTTAAATTAAGATGCTCCACAGAGACATTCCG-3' |
| coku80CR | 5'-TCTCTGTACAGTCTCTGACATGCTGACATTCCG-3' |
| copyRF | 5'-TAGCGGCCGCGGATACGCTCTC-3' |
| copyRF | 5'-CCTTAATACGGTACACTCCATACCCCTACTCA-3' |
| Primers used for the Aaarg8 disruption |
| AaargBF5 | 5'-GGCGTGATTTCTTCTGCTC-3' |
| AaargB52 | 5'-GAGACATTGTCTGCGCGGCTGGAGTCGAGTCG-3' |
| AaargBF3 | 5'-GAGTGGAAAGAATGAAAGATGCAGTAGGACCGAG-3' |
| AaargBF3r | 5'-GGGAGACATGGCTCTCCTAC-3' |
| npyrF3 | 5'-GACCTCGACGGCCGACCCGCAACTGCTCGTC-3' |
| npyrR3 | 5'-CTCGGTGGATCAGTCTGCTGACATTCCG-3' |
| marq5F | 5'-GGCGGCCGCGGAGCTC-3' |
| marq5R | 5'-GACCACCCCGTCTGGCAATGCGACATGCTC-3' |
| marq5R | 5'-GGCAGCGATCCAGAGGAGGAGAATGCTGCCAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
| marq5F | 5'-CCGATTAGGCGGATACGCGGATGC-3' |
| marq5R | 5'-GAGAGCATTGTCTGCGAGATGCAGTAGGACCGAG-3' |
| marq5F | 5'-CTGGAAGGCTTCAATGCGCGGCGAACAGATGCTC-3' |
| marq5R | 5'-GACAAAATCTCCCTGCTGCTC-3' |
The *A. aculeatus* ornithine carbamoyltransferase gene (*AaargB*) was also cloned from the *A. aculeatus* genome by the same strategy. The *AaargB* segment was amplified with the primers *AaargBF3* and *AaargBR2*. After performing Southern blot analysis and colony hybridization using the *AaargB* segment as a DNA probe, the *AaargB* open reading frame (ORF) with flanking regions, an approximately 6-kb *Hin*dIII-digested fragment, was cloned into pBS II KS (+), yielding pAaargB.

**Transformation of *A. aculeatus***

The transformations were performed by following the protoplast–PEG method as described previously (Adachi et al. 2009). The strains were propagated at 30°C in MM supplemented with the appropriate nutrients.

**Gene disruption and marker recycling**

The linear DNA fragments used for the gene deletion of *Aaku80* followed by marker recycling were generated by fusion PCR as described previously (Akada et al. 2006). To construct the *Aaku80* deletion cassette, the 5′ and 3′ regions of the *Aaku80* gene, which are required for HR, were amplified from *A. aculeatus* genomic DNA using the primer pairs *aku80AF1* and *aku80AR2* for the 5′ region and *aku80BR1* for the 3′ region. The 3′ flanking region of the *Aaku80* gene, which formed DRs, was amplified from *A. aculeatus* genomic DNA using the primer pair *aku80CF1* and *aku80CR1*, and the fused product was subcloned into the *Eco*RV site of pBS II KS (+), which yielded pDku80. The *Aaku80* deletion cassette was amplified with the primers *aku80AF1* and *aku80BR1*, and the fused product was introduced into the *pyrG13* strain using the protoplast–PEG method. The deletion of endogenous *Aaku80* was confirmed by Southern blot analysis using DNA probes that were amplified with the primers *ku80R4* and *aku80BR1* using pAaku80 as a template.

**FOA-resistant strains by marker recycling**

FOA-resistant strains by marker recycling were spontaneously isolated from 1 × 10⁴ to 10⁶ *A. aculeatus* transformant conidia in MM with 1 mM FOA and 0.4 mM uridine. These conidia were purified by repeating monospore isolation twice on the plates to obtain the conidia of homokaryons. Their growth was examined on plates with or without 8 mM uridine.

To develop a strain enabling a reversible impairment of *Aaku80*, an *Aaku80* disruption cassette 2 was constructed. The 5′- and 3′-regions of the *Aaku80* gene were amplified from *A. aculeatus* genomic DNA using the primer pairs *coku80NF* and *coku80NR* for the 5′ region and *pyrG13* as a template.

**Table 1 List of primers used in this study (Continued)**

| Primer | Sequence |
|--------|----------|
| AaargB3f3 | 5′-GAGTGGAAATGTGTAACGGATTGCATGGACCCGAG-3′ |
| AaargB3r | 5′-GGGGACATGGCTTCCTAC-3′ |
| DxlnR-F1 | 5′-GTCGACCACCCGGATACGATCGCCA-3′ |
| DxlnR-R2 | 5′-CAACACACAGTGAGACCCACTTCCTC-3′ |

![Figure 1 Southern blot analysis of the *Aaku80*-deletion mutants.](image)
and coku80CF and coku80CR for the 3' region. The second intronic region was amplified with the coku80NR and coku80CR primers. The AnpyrG fragment was amplified using pPL6 as a template with the primer pair copyR and copyR. The 5'-side of Aaku80 digested with EcoRV and NotI and AnpyrG digested with NotI were inserted into the EcoRV site in pBS II KS (+) to yield pCO5P. The 3'-side of Aaku80 digested with PacI, and XbaI was ligated to the PacI and XbaI sites on pCO5P to yield pCO5P3. The Aaku80 disruption cassette 2 was amplified with the primers coku80NF and coku80CR using pCO5P3 as a template. The fragments were introduced into the pyrG13 strain using the protoplast–PEG method. The disruption of endogenous Aaku80 was confirmed by Southern blot analysis using DNA probes that were amplified with the primers mrarg5F and mrarg5R, mrarg3F and mrarg3R, and mrarg3F3 and mrarg3R3 for the 5' DNA probe and mrarg3F1 and mrarg3R for the 3'-DNA probe using pAaargB as a template.

Deletion of the A. aculeatus xlnR gene, encoding a gene-specific activator controlling cellulase and hemicellulase gene expression, was performed as described previously (Tani et al. 2012). The xlnR deletion in the selected strains was confirmed by analyzing each genome using Southern blot analysis and PCR using the primers DxlNR-F1 and DxlNR-R2.

Results
Deletion of the Aaku80 gene and recycling of the AnpyrG marker
As a first step toward developing HR and marker-recycling systems in A. aculeatus, Aaku80 was cloned. Sequence analysis and a homology search revealed that the Aaku80 gene comprised a 2,711-bp ORF interrupted by nine introns and encoding 726 amino acid residues with a calculated molecular mass of 81,088 Da (DDJB Acc. no. AB741874). The Aaku80 gene in pyrG13 (Aaku80−, pyrG+) was replaced with AnpyrG using the Aaku80 deletion cassette (Figure 1a). Forty transformants were obtained by selection on a uridine-free medium. Southern blot analysis confirmed that one (2.5%) of these transformants, designated R1, had one copy of the deletion cassette inserted into the Aaku80 locus (Figure 1b, lane 2). Next, we tried to excise the AnpyrG-containing fragment sandwiched by a pair of 1-kb DRs to reuse AnpyrG as a selection marker, as is commonly done in Saccharomyces cerevisiae (Akada, et al. 2006). Conidia (approximately 1 × 10⁶) were spread onto a 5-FOA plate, and 20–30 colonies on average that should have been 5-FOA-resistant appeared after 5 d of cultivation, while it took more than 10 d for resistant mutants to emerge on 5-FOA plates by spontaneous mutation. After mono-spore isolation, more than 90% of the isolates showed uridine auxotrophy. Southern blot analysis showed that the expected excision event of intramolecular HR occurred in 9 of 10 uridine auxotrophs (Figure 1b, lane 3). One strain (Aaku80−, pyrG−) was designated as strain MR12.

Characteristics of the Aaku80 disruptant
The growth and sporulation of MR12 on MM were indistinguishable from those of the control strain (pyrG13), but its growth on 0.05% methyl methanesulfonate (MMS) was poorer than that of pyrG13 (Figure 2). This result agreed well with previous reports that ku disruption in Neurospora

Tani et al. AMB Express 2013, 3:4
http://www.amb-express.com/content/3/1/4
crassa or Aspergillus fumigatus increased sensitivity to the DNA-damaging reagent (Ninomiya et al. 2004; da Silva Ferreira et al. 2006).

To compare the HR frequency at the AaargB locus in pyrG13 with that in MR12, the AaargB gene was cloned. Sequence analysis and a homology search revealed that AaargB comprised a 1,089-bp ORF with no introns and encoded 362 amino acid residues with a calculated molecular mass of 38,980 Da (DDBJ Acc. no. AB741875). The AaargB gene was replaced with a selection marker using the AaargB deletion cassette 1 in pyrG13 and MR12 to elucidate the gene targeting frequencies. Arginine auxotrophs were found in one of 32 transformants of pyrG13 (targeting frequency of 3%) and in 29 of 31 transformants of MR12 (94%). Southern blot analysis showed that all arginine auxotrophs derived from MR12 had a single copy of the cassette at the target locus by double HR (data not shown).

**Figure 2. Sensitivity of pyrG13 (pyrG) and MR12 (Aaku80, pyrG) strains of Aspergillus aculeatus to methyl methanesulfonate.** Conidia were spotted and grew on minimal medium with or without methyl methanesulfonate (MMS) for 5 d.

**Construction of pyrG argB double-auxotrophic host and effect of DR length on intramolecular homologous recombination**

The arginine auxotrophic host was generated with point mutations to retain the argB ORF as the target of homologous transformation. The argB disruption cassette 2 was designed to insert the nucleotides G and A behind the 25th G and 27th T from a translation start site, which yielded a NheI recognition site and Ala9Gly mutation followed by a stop codon, respectively (Figure 3a). The disruption cassettes were also designed to harbor different length DRs (20, 98, or 495 bp) and the AnpyrG selection marker to elucidate the effect of DR length on intramolecular HR. The cassettes were introduced into MR12 and yielded 15, 10, and 20 uridine prototrophic transformants, respectively. From these, 11 (73%), 10 (100%), and 17 (85%) arginine auxotrophs were isolated, respectively. Strains having a single copy of the insert were selected to give the mTA20, mTA98, and mTA495 strains harboring the DRs of the indicated lengths (Figure 3b, lanes 2–4). Conidia (1 × 10⁶) of mTA495 were spread on plates containing 5-FOA, uridine, and arginine, and 120 colonies developed. Ten 5-FOA-resistant strains were selected for further analysis and found to be uridine auxotrophs. Southern blot analysis confirmed that AnpyrG was excised by intramolecular HR from the argB locus in nine of the ten uridine auxotrophs (Figure 3c, lanes 2–4). Thus, an A. aculeatus host with double selection markers was successfully constructed. The frequency of the emergence of the uridine auxotroph by intramolecular HR from mTA495 was calculated to be 1.1 × 10⁻⁴. Those in mTA98 and mTA20 were calculated to be 7.0 × 10⁻⁶ and 3.0 × 10⁻⁶, respectively (Table 2). These results indicated that excision frequency was correlated with longer DRs. Although the frequency of AnpyrG excision in mTA20 was almost equal to the frequency of isolating the uridine auxotroph by spontaneous mutation from the wild-type strain, these events could be distinguished by culture duration, because the 5-FOA resistant strains resulting from intramolecular HR appeared earlier than did those by spontaneous mutations.

**Mitotic stability analysis of DNA with direct repeat sequences**

Subculturing the mTA495 strain five times on MM with uridine did not increase the frequency of emergence of 5-FOA resitants (data not shown). Furthermore, intramolecular recombination between DRs was investigated by Southern blot analysis using chromosomal DNA isolated from the mTA495 strain grown in liquid MM with or without uridine for 5 d. The hybridization patterns of the AaargB locus in the mTA495 were the same as shown in Figure 3b (lane 4). The same results were obtained in mTA20 (lane 2) and mTA98 (lane 3). These data indicated that the short DRs were stably maintained in the transformants.

**Insertion of AnpyrG into the second intron of Aaku80 enables the disruption and restoration of Aaku80**

Establishing a simple method to restore the function of Aaku80 after deleting specific genomic regions would be an advantage in filamentous fungi without a feasible genetic segregation technique, such as A. aculeatus. Therefore, we designed the Aaku80 disruption cassette 2, which introduced
the AnpyrG gene into the second intron of the Aaku80 gene (Figure 4a). Because the direction of AnpyrG transcription is the same as that of Aaku80, we presumed that the termination of AnpyrG transcription yielded immature transcripts of Aaku80 and resulted in the loss of Ku80 function. Furthermore, we expected that a functional Ku80 protein would be produced even if a frameshift mutation occurred between the repeats because the insert was in an intron in Aaku80. The pyrG13 strain was transformed with the Aaku80 disruption cassette 2 and yielded transformants harboring the cassette integrated as a single copy, namely coku80, into the Aaku80 locus by homologous integration (Figure 4b, lane 2). The coku80rec strain was isolated by excision of the AnpyrG gene by marker recycling at the frequency of $3.3 \times 10^{-5}$ (Figure 4b, lane 3). Sequence analyses of Aaku80 of five independently-isolated coku80rec strains confirmed that AnpyrG was excised between DRs without any mutations.

Growth and sporulation of the coku80 and coku80rec strains on MM were indistinguishable from those of MR12.
The 5-fluoroorotic acid (5-FOA) resistant strains were isolated from 1.0 × 10⁶ conidia.

Table 2 Frequencies of intramolecular homologous recombination between direct repeats (DRs) of different lengths

| Strain | mrTA20 | mrTA98 | mrTA495 |
|--------|--------|--------|---------|
| Length of DR (bp) | 20 | 98 | 495 |
| No. of 5-FOA-resistant strains | 6 | 14 | 120 |
| No. of resistant strains analyzed | 6 | 6 | 10 |
| No. of uridine auxotrophs | 6 | 4 | 9 |
| No. of homologous recombination events between DRs | 3 | 3 | 9 |

The 5-fluoroorotic acid (5-FOA) resistant strains were isolated from 1.0 × 10⁶ conidia.

(Aaku80°, pyrG°) and pyrG13 (pyrG°), but growth of coku80 on 0.05% MMS was as poor as that of MR12. However, both coku80rec and pyrG13 grew normally on 0.05% MMS (data not shown). Transformation of pyrG13, MR12, coku80, and coku80rec with the xlnR deletion cassette yielded 1 (of 51 transformants; 2%), 31 (of 46; 67%), 26 (of 35; 74%), and 1 (of 38; 3%) xylan-utilization-deficient mutants. AnpyrG insertion into the second intron of Aaku80 successfully disrupted Aaku80 function, which was restored by intramolecular HR between 20-bp DRs. Furthermore, the coku80 strain stably maintained the AnpyrG gene after 10 generations and yielded 17 xylan-utilization-deficient mutants out of 21 transformants (81%). These data indicate that coku80 is a feasible host for genetic analysis of interesting genes in the Aaku80 functional background.

Discussion

In Aspergillus, auxotrophic mutants, such as pyrG, niaD (nitrate reductase), or sC (ATP-sulhydrylase)-deficient mutants, can be simply isolated because they are positively selected in the presence of the counter-selective compounds (Arst 1968; Daboussi et al. 1989; Goosen et al. 1987). To advance molecular biology in A. aculeatus, we designed and validated a HR method for gene replacement by deleting the Aaku80 gene. The Aaku80 deletion drastically increased the gene-targeting frequency from 3% to 94% and enabled the construction of double auxotrophic mutants (Aaku80, argB°, pyrG°). This drastic increase in gene-targeting frequency reduced the labor required to isolate auxotrophic mutants, such as an arginine auxotroph, without the use of counter-selective compounds. The 94% targeting frequency in A. aculeatus was high enough for practical use in gene replacement. However, the targeting frequency for approximately 1-kb homologous sequences was lower than in N. crassa (Ninomiya et al. 2004). Because homology length is critical to the homologous integration frequency, A. aculeatus might require a longer homologous region for gene targeting.

Because the number of marker genes is limited, establishing a marker-recycling technique is necessary for the consecutive insertion of gene expression cassettes or multiple-gene deletions. For marker rescue, the cre-loxP recombination system of bacteriophage P1 has been shown to mediate efficient recombination between 34-bp loxP sites flanking a marker gene in yeast and filamentous fungi, resulting in excision of the marker gene (Krapmann et al. 2005; Sauer 1987). Gene deletion by HR between the loxP sites results in marker removal, leaving behind a single repeat at the deleted gene locus. Therefore, developing a self-cloning technique to breed genetically-modified filamentous fungi is important. To achieve this aim, the double-marker enrichment technique was adopted for target-gene deletion in A. fumigatus, Aspergillus awamori, A. nidulans, and Leptosphaeria maculans (d’Enfert 1996; Gardiner and Howlett 2004; Krapmann and Braus 2003; Michielse et al. 2005). In this strategy, a second selectable marker is added to the gene deletion cassette to distinguish between HR and non-homologous recombination. Upon HR, the second selection marker is lost, whereas after integration by non-homologous recombination, transformants carrying both selectable markers are obtained. Alternatively, once a mutant with a two-way (positive and negative) selectable marker such as pyrG is isolated, marker recycling enables not only consecutive gene deletion, but also consecutive gene insertion without leaving behind an exogenous DNA fragment. Although 50–100 bp of DRs were sufficient to facilitate gene-replacement cassette integration at the

**Table 2 Frequencies of intramolecular homologous recombination between direct repeats (DRs) of different lengths**

**Figure 4 Southern blot analyses of the Aaku80-disruption mutants.** (a) Diagram of Aaku80 disruption cassette 2. The candy-striped bars indicate the second intron of Aaku80. Results of Southern blot analyses using genomic DNA digested with (b) BgII (BII) and (c) PstI (P). Lanes 1, 2, and 3 are MR12, coku80, and coku80rec strains, respectively.
homologous locus at a gene replacement efficiency of 50–100% in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Bahler et al. 1998; Wach et al. 1994), longer DRs were thought to be required in filamentous fungi (Hynes 1996; Maruyama and Kitamoto 2008; Nielsen et al. 2008; Ninomiya et al. 2004). However, our results indicated that the correct excision event could occur even with a 20-bp DR, although the frequency was relatively low. The observation that a shorter DR works sufficiently for correct excision should help to design a deletion cassette for marker recycling. Using this method, we are constructing an *A. aculeatus* host with multiple selectable markers to enable the breeding of filamentous fungi that produce several different cellulases to hydrolyze cellulosic substances with high efficiency.

Despite whole-genome sequencing, the roles of many gene products in fungi remain unknown, in part because gene deletion may not alter the phenotype when more than one paralog exists in the genome. The technique developed in this paper could be a useful tool for postgenomic research.

**Competing interests**

The authors declare that they have no competing interests.

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