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Development of a novel quadruple auxotrophic host transformation system by *argB* gene disruption using *adeA* gene and exploiting adenine auxotrophy in *Aspergillus oryzae*

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Abstract

We previously designed a triple auxotrophic host-vector system in *Aspergillus oryzae* by isolating red-colored adenine auxotrophic mutants upon UV mutagenesis of a double auxotrophic host (*niaD*$^-$ *sC*$^-$). In the present study an effort to exploit this system and construct a novel quadruple auxotrophic host was made by disrupting the *argB* gene involved in arginine biosynthesis. The *argB* gene-disruption cassette was generated by fusion PCR, which required only two steps of PCR to insert the selectable marker, *adeA*, into the target *argB* gene. The chimeric DNA fragment was transformed into the triple auxotrophic strain (*niaD*$^-$ *sC*$^-$*adeA*$^-$) and the *argB* disruptants were obtained with a high rate of efficiency (approximately 40%). The *argB* disruptants were characterized by normal colony color and reversal of arginine auxotrophy by introduction of the wild-type *argB* gene. Quadruple auxotrophic strains (*niaD*$^-$ *sC*$^-$Δ*argB adeA*$^-$ or *niaD*$^-$ *sC*$^-$Δ*argB adeB*$^-$) were subsequently isolated upon UV mutagenesis of the triple auxotrophic strain (*niaD*$^-$ *sC*$^-$Δ*argB*) followed by screening of red-colored colonies for adenine auxotrophy. The results obtained showed that the *adeA* gene served as an efficient selection marker in developing a novel host-vector system with quadruple auxotrophy in *A. oryzae*, thus providing a powerful tool to breed multiple auxotrophic mutants from a deuteromycete wherein sexual crossing is impossible.

Keywords: *Aspergillus oryzae*; Transformation; *argB*; Quadruple auxotrophy; Adenine; UV mutagenesis; Red colony

1. Introduction

Filamentous fungi, by virtue of their ability to secrete large amounts of hydrolyzing enzymes are being used as hosts for heterologous protein production. However, proteases have often remained a hurdle for efficient production of heterologous proteins in filamentous fungi [1]. Therefore, eliminating important proteases may be a better alternative to improve the yield of heterologous proteins as demonstrated in *Aspergillus niger* strains with double and triple disruptions of protease-encoding genes [2]. Recently, forced expression of ER chaperone genes and activation of unfolded protein response (UPR) pathway also improved production level of heterologous proteins [3–5].

Among the filamentous fungi, *Aspergillus oryzae* is regarded as an attractive host for heterologous protein production. The long history of its usage (more than 1000 years) in Japanese traditional food fermentations such as for sake, *miso* and soy sauce manufacturing

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supports its well-established culturing technique (i.e., solid-state fermentation) and its GRAS (generally regarded as safe) status [6]. Further, the development of improved promoter systems facilitated higher yield of target proteins [7]. Moreover, recent progress in genomics of *A. oryzae* is expected to increase the number of genes to be investigated by molecular genetic analyses [8]. In order to breed favorable hosts for heterologous protein production, manipulation of several bottleneck genes relating to proteases, secretory pathway, cell polarity establishment and metabolism is necessary. However, lack of sexual life cycle in *A. oryzae* has impeded advances in genetic breeding. In spite of the development of *A. oryzae* transformation system using several selectable markers [9–15] including double and triple auxotrophic hosts [13,15], no attempt to disrupt multiple genes has been reported.

Since approaches to develop more selectable markers in *A. oryzae* are bound with difficulties in enrichment of auxotrophic mutants or otherwise related to antibiotic resistance, in a previous study we took advantage of an easier method of screening based on the consequence of *ade1*, and/or *ade2*, mutation in the adenine biosynthetic pathway that resulted in red-colored colonies in yeast [16,17]. This method of screening facilitated the isolation of triple auxotrophic hosts (*niaD*$^-$ *sC*$^-$ *adeA*$^-$; *niaD*$^-$ *sC*$^-$ *adeB*$^-$) from a double auxotrophic strain (*niaD*$^-$ *sC*$^-$) [15]. In the present study we developed a novel transformation system with quadruple auxotrophy in *A. oryzae* by disrupting the *argB* gene with the *adeA* gene and then utilizing the easy colony-color screening method to reconfer adenine auxotrophy after UV mutagenesis. Moreover, the *argB* gene encoding the ornithine transcarbamylase (OTCase) has earlier been used as one of the most convenient markers for gene manipulation in *Aspergilli* [18,19]. We herein discuss the results obtained in this regard and on the significance of this transformation system being a powerful tool to breed more auxotrophic strains of *A. oryzae* and also other deuteromycetes wherein sexual crossing is impossible.

2. Materials and methods

2.1. Strains and growth media

Aspergillus oryzae RIB40 (wild-type) and NSR13 (*niaD*$^-$ *sC*$^-$ *adeA*$^-$) [15] strains were used for amplification and disruption of the *argB* gene, respectively. NS4 (*niaD*$^-$ *sC*$^-$) [13] strain was used for auxotrophy experiments. DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_20$, pH 5.5) and M medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 2% glucose, pH 5.5) supplemented with 0.15% methionine, 0.01% adenine and/or 0.1% arginine were used for growth and auxotrophy experiments, respectively.

2.2. Cloning of A. oryzae argB gene

In order to amplify *A. oryzae argB* gene by PCR, the primers, argB-F(273) (5′-gaggagtaaaggggtggattcgga-3′) and argB-R(2349) (5′-gggttgttggccttgttttgtcgg-3′) were designed based on the sequence in the database [20]. Using genomic DNA of RIB40 as a template, 2.1 kb *argB* fragment was amplified by *Pfx* DNA polymerase (Invitrogen, USA), which was then tailed with dATP at 3′-ends using *Ex Taq* DNA polymerase (TaKaRa, Japan). This procedure facilitated cloning of the *argB* fragment into pT7Blue vector (Novagen, USA), resulting in generation of pArgB (Fig. 2(a)).

2.3. Generation of the argB gene disruption cassette by fusion PCR

The strategy for fusion PCR is shown in Fig. 1(a). Using the genomic DNA of *A. oryzae* RIB40 as a template, 2.0 kb *adeA* gene was amplified with the primers adeA-F (5′-ccgtcatgtccaggaagataggtcag-3′) and adeA-R (5′-ctgcgcaacagcatacgagtccacag-3′). Two DNA fragments including the first and the latter halves of 2.6 kb full-length *argB* gene were generated using the primers, argB-A (5′-gccgactaaccgaaacatt-3′) and argB-B (5′-ctgacctatcttcctggacatgacgg-tatcctggttctggtactgc-3′) for the first half of the *argB* gene, and argB-C (5′-ggactcg-tatgctgttgcgca-gacatccaatgtttctgggc-3′) and argB-D (5′-ggacggagtaactggaaaga-3′) for the latter half of the *argB* gene, respectively. The underlined sequences in argB-B and argB-C were designed to overlap the sequences of adeA-F and adeA-R, respectively. Additionally, a 31 bp gap was created between the first and latter halves of the *argB* gene using the argB-B and argB-C primers. PCR for amplification of the *adeA* gene and the two fragments of the *argB* gene was carried out using *Pfx* DNA polymerase. The three DNA fragments were then mixed and fusion PCR was performed by *LA Taq* DNA polymerase (TaKaRa) according to the manufacturer's instruction. The PCR reaction was performed as follows: denaturation at 94 °C for 2 min; followed by 30 cycles of 94 °C for 30 s, 45 °C for 30 s and 68 °C for 5 min; with a final extension at 68 °C for 5 min (the temperature from 45 to 68 °C was gradually raised at 1 °C/10 s). The gene disruption construct (4.6 kb) was cloned into pT7Blue vector, generating pARGA. Using pARGA as a template, PCR was performed with outermost primers, argB-A and argB-D, and *LA Taq*, and the amplified DNA fragment was used for transformation of *A. oryzae*.

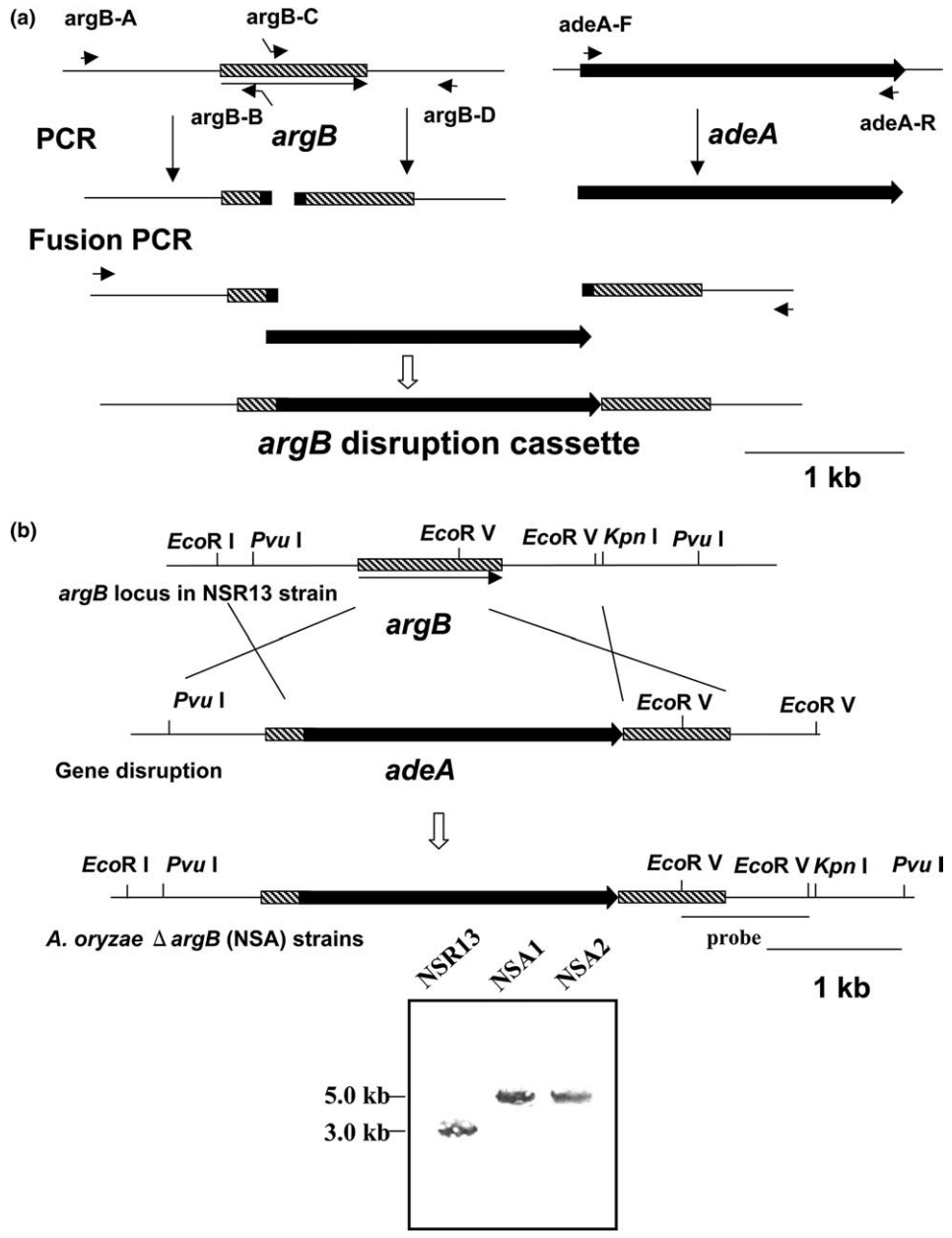

Fig. 1. Disruption of *A. oryzae argB* gene. (a) Construction of the DNA fragment for the *argB* gene disruption by fusion PCR. The 5′ends of primers argB-B and argB-C were designed to be complementary to primers adeA-F and adeA-R, respectively. There is a 31 bp gap between the locations of the primers argB-B and argB-C in the *argB* gene. See Section 2.3 for details. (b) Southern blot analysis of the *argB*-disruptant. The *Eco*RV-digested 0.8 kb DNA fragment (see Section 2.4) was used as probe. Genomic DNAs of the parent strain and transformants were digested with *Pvu*I. Lane 1, NSR13 (parent); lanes 2 and 3, NSA1 and NSA2.

2.4. Disruption of the argB gene in A. oryzae

Transformation of *A. oryzae* for the disruption of the *argB* gene was performed as described by Maruyama et al. [21] with minor modifications. Conidia of the NSR13 strain cultivated on M agar medium containing methionine and adenine for 3 days were collected, and cultivated in 100 ml DPY liquid medium for approximately 17 h. Protoplasts were prepared from the mycelial cultures by Yatalase (TaKaRa) treatment for 3 h at 30 °C, and transformed with the amplified PCR product as described in Section 2.3. Transformants were obtained on M agar medium containing 0.15% methionine and 0.1% arginine. After isolation, *argB*-disruptants were identified by PCR and Southern blot analyses. The primer argB-P (5′-tgaattccatccgctgtggc-3′) designed based on the upstream region to the left of primer argB-A was used for PCR analysis. For Southern analysis the genomic DNA from the parent strain and the transformants was separated on a 0.8% agarose gel by

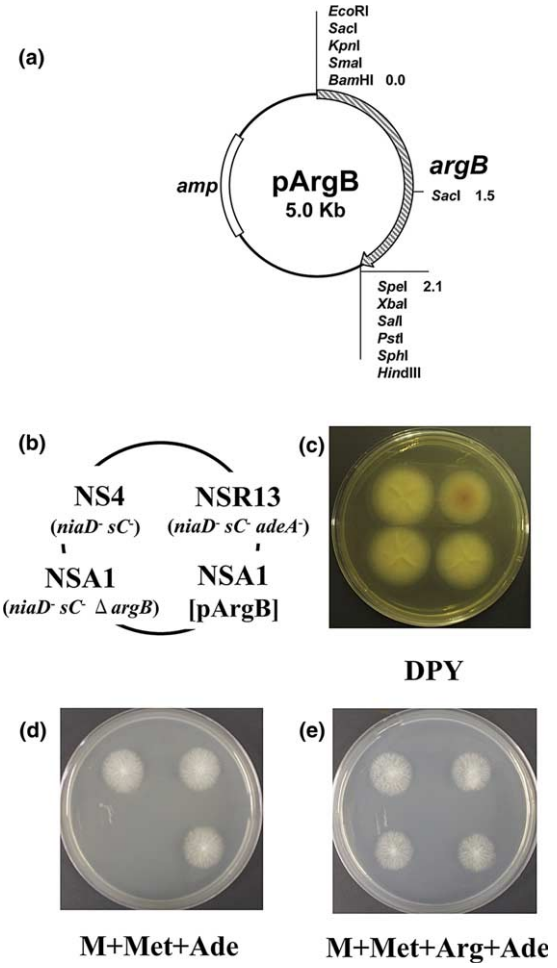

Fig. 2. Growth of the *argB* disruptant. (a) Plasmid used for transformation of the *argB* disruptant. (b) Growth of the *argB* disruptant and its restored phenotype. The representative strains are shown in the circle. The wild-type strain (NS4), the parent strain (NSR13), the *argB* disruptant (NSA1), and the NSA1 strains transformed with pArgB were grown in DPY medium (c), M + Met + Ade (d), M + Met + Arg agar media (e) for 3 days at 30 °C.

electrophoresis after digestion with *Pvu*I, and then transferred to Hybond N^+ membrane (Amersham Biosciences, USA). ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system (Amersham Biosciences) and LAS-100plus luminescent image analyzer (Fuji Photo Film, Japan) were used for Southern blot analysis. Plasmid pARG that contained 2.7 kb *Eco*RI–*Kpn*I fragment of the *argB* gene (Fig. 1(b)) in pUC118 was digested with *Eco*RV and the 0.8 kb fragment was used as a probe.

2.5. Isolation of adenine auxotrophic mutants

Two milliliters of the suspension containing 8×10^6 conidia of *A. oryzae* NSA1 (*niaD*$^-$ *sC*$^-$ Δ*argB*) was transferred into a sterile Petri dish (diameter: 5.3 cm) and gently stirred using a magnetic stirrer while irradiating with a 15 W UV lamp fitted at a height of 53 cm in a safety cabinet SCV-ECIIA (Hitachi, Japan) for time periods of 3 and 6 min, which kept the survival rates at approximately 50% and 20%, respectively. After incubation in the dark for 1 h, the conidia were then spread on DPY agar medium containing 0.25% Triton X-100 in order to obtain compact colonies (approximately 400 colonies per plate). Red-colored colonies were selected and their adenine auxotrophy was examined on M agar medium supplemented with 0.15% methionine and 0.1% arginine in presence or in absence of 0.01% adenine. To investigate the mutated site in *adeA*, the *adeA* gene inserted into the *argB* locus was amplified with the primers (argB-A and argB-D) using genomic DNA of the *adeA* mutants as a template. The amplified fragment was sequenced by ABI PRISM™ 310NT Genetic Analyzer (Applied Biosystems, USA).

3. Results

3.1. Construction of the argB gene disruption cassette by fusion PCR

In order to obtain the *argB* gene disruption cassette, a fusion PCR strategy was devised according to Kuwayama et al. [22] (Fig. 1(a)). This method was based on the fusion of three DNA fragments (the *adeA* gene fragment between the 2 halves of the *argB* gene) by a convenient two step PCR to obtain the *argB* gene disruption construct. Briefly, in the first step the 2.0 kb DNA fragment of the *adeA* gene, as well as the two DNA fragments consisting of the first and the latter halves of the 2.6 kb *argB* gene, were amplified separately by PCR using *A. oryzae* RIB40 genomic DNA as a template. In the second step, the three DNA fragments were connected together by a fusion PCR reaction, utilizing the short overlapped sequences at their terminal regions. Finally, a 4.6 kb *argB* gene disruption cassette was amplified and its sequence was confirmed by DNA sequencing prior to transformation.

3.2. Isolation and characterization of arginine auxotrophic strains of A. oryzae

The *argB* disruption cassette constructed by fusion PCR was transformed into *A. oryzae* NSR13. Among the 5 transformants obtained 2 of them designated as NSA1 and NSA2 (*niaD*$^-$ *sC*$^-$Δ*argB*) were confirmed to be arginine auxotrophic strains. Southern analyses revealed a 3.0 kb band in the parental strain (NSR13), while the *argB*-disruptants (NSA1 and NSA2) showed an *adeA* -inserted 5.0 kb band (Fig. 1(b)). The *argB* disruption in the 2 transformants was also verified by PCR using primers argB-P and argB-D to amplify a 4.6 kb *argB*-disrupted DNA fragment in comparison to the

2.6 kb wild-type *argB* fragment (data not shown). To verify arginine auxotrophy, the parental strains (NS4 and NSR13) and the transformed strain (NSA1) were inoculated on complete (DPY; Fig. 2(c)) or adenine-containing minimal medium in the absence or presence of arginine (Fig. 2(d) and (e)). While the parental strains, NS4 and NSR13, grew irrespective of the growth media, the NSA1 strain failed to grow in the absence of arginine, demonstrating arginine auxotrophy (Fig. 2(d)). In order to perform complementation test, a plasmid, pArgB, was constructed using pT7Blue based vector as described in Section 2.2 and shown in Fig. 2(a). The NSA1 transformant with pArgB showed apparently normal phenotype similar to the NS4 and the NSR13 strains even in the absence of arginine (Fig. 2(d)). This result indicated that the wild-type *argB* gene complemented arginine auxotrophy in the NSA1 strain. In addition the NSA1 strain exhibited normal colony color on DPY agar medium when compared to a red colony color of the NSR13 strain (Fig. 2(c)), demonstrating that adenine auxotrophy was restored by transformation with the *adeA* gene.

3.3. Screening of adenine auxotrophic mutants for breeding quadruple auxotrophic hosts in A. oryzae

We had earlier reported a distinctive colony selection method based on the colony color for the isolation of adenine auxotrophic mutants from *A. oryzae* [15]. In the present study, a similar strategy to isolate adenine auxotrophic mutants from the NSA1 strain was attempted in order to breed quadruple auxotrophic mutants of *A. oryzae*. Conidia of the NSA1 strain were subjected to UV irradiation as described in Section 2.5. After 3-day cultivation on DPY agar medium, 10 red-colored colonies (designated hereafter as NSAR strains) were selected from the surviving colonies. Among the mutants, the NSAR1 strain was isolated at a survival rate of 50% while the others were isolated at about 20% survival rate. In order to verify if the isolated mutants exhibited adenine auxotrophy growth test was performed in the presence or absence of adenine (Fig. 3). While all the strains grew well in DPY medium (Fig. 3(b)), the NSAR1 and the NSAR2 strains failed to grow in arginine-containing minimal medium lacking adenine (Fig. 3(c)) and supplementation with adenine restored their growth (Fig. 3(d)), indicating the requirement of adenine for their survival. In order to reconfirm the adenine auxotrophy of the mutants, plasmids pAdeA or pAdeB [15] were transformed into the respective strains and verified for growth in the absence of adenine. As shown in Fig. 3(c) the *adeA* gene and the *adeB* gene complemented the NSAR1 and the NSAR2 strains, respectively. Adenine auxotrophy of the NSAR1 and the NSAR9 strains was complemented by *adeA* gene, while the other eight strains were complemented by *adeB* gene (data not shown). Transformation efficiency of the strains ranged between 1–3 colonies and 1–4 colonies per microgram of transforming DNA containing *adeA* and *adeB* gene, respectively.

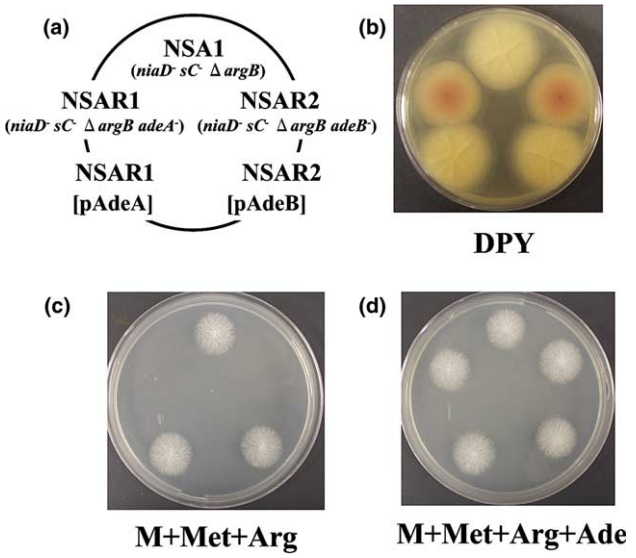

Fig. 3. Growth of quadruple auxotrophic mutants. The representative strains are shown in the circle (a). The parent strain (NSA1), the quadruple auxotrophic mutants (NSAR1 and NSAR2), and NSAR1 and NSAR2 transformed with the *adeA* gene in pAdeA and *adeB* gene in pAdeB, respectively, were grown on DPY (b) and M + Met + Arg agar media with/without adenine for 3 days at 30 °C (c and d). The NSAR1 and the NSAR2 strains grew as red-color colonies due to their adenine auxotrophy on DPY agar medium and failed to grow on M + Met + Arg devoid of adenine.

3.4. Characterization of the adeA and the adeB gene mutations in the quadruple auxotrophic strains

Since the quadruple auxotrophic strain, NSAR1, was isolated at a higher survival rate (approximately 50%), it is expected to have mutations only in the *adeA* gene and not on any other genes. It was, therefore, of interest to examine the mutated sites in the *adeA* gene of this strain. Nucleotide sequence of the *adeA* gene inserted into the *argB* locus in the NSAR1 strain revealed a frameshift mutation in the *adeA* gene due to the deletion of the single T located at the 787th nucleotide from the start of ORF. Additionally, a G_{785}-to-T transversion mutation which resulted in substitution of Gln with His in the strain was noted. On the other hand, in the case of the *adeB* gene of the NSAR2 strain a frame shift mutation occurred due to the deletion of the 9th nucleotide (C) from the start of ORF. We have earlier reported both a deletion of a single base pair and a substitution mutation in the adenine auxotrophic NSR strains during the isolation of triple auxotrophic strains of *A. oryzae* [15]. This observation is also supported by studies on UV induced mutations in *Escherichia coli* by Miller [23] which

primarily resulted either in a substitution or elimination of a single base pair.

A negative control experiment of inoculating the NSAR1 strain without transformed DNA on selective media indicated that reversion did not occur (data not shown) and, hence, it was selected as a quadruple auxotrophic host for future experiments. Separate transformation and growth experiments were also performed to verify the quadruple auxotrophic nature of the NSAR1 strain with plasmids harboring *niaD*, *sC*, *argB*, or *adeA* genes and with growth on media containing appropriate supplements. As expected, transformants were successfully obtained using the marker genes *sC*, *argB*, and *adeA*. However, due to higher background of colonies in the medium containing both arginine and adenine, it is recommended that the *niaD* gene be used as a marker only after transformation with the *argB* and the *adeA* genes (data not shown). These observations show that the NSAR1 strain can be utilized as a suitable transformation host with quadruple auxotrophy.

4. Discussion

In the present study we employed a fusion PCR strategy for disruption of the *argB* gene. Although the fusion PCR-based construction of a gene disruption fragment has been established in yeast [24,25] and *Dictyostelium discoideum* [22], this is a novel approach in filamentous fungi. While it is a faster and efficient method in comparison to other methods that use DNA ligase and restriction enzymes, it is also possible to attach long flanking regions to any selectable marker and delete any part of the entire sequenced ORF. This method allows rapid construction of gene disruption fragments and, therefore, can be a very useful tool to conduct a genome-wide analysis of gene function in *A. oryzae*.

Disruption of the *argB* gene in *A. oryzae* with the *adeA* gene revealed that a double homologous integration occurred at 40% in the *argB* locus. Repetition of similar transformations revealed the efficiency to be between 33% and 40%. In comparison to other transformation markers used in *A. oryzae*, the targeting efficiency of the *adeA* gene was higher than *A. nidulans sC* gene (3.2 kb), which has earlier been used for gene disruption in *A. oryzae* [21]. This is presumably due to the smaller size of the *adeA* marker (2.0 kb) when compared to other known selectable markers. Recently, we have succeeded in disruption of the *pepA* gene encoding a putative extracellular acid protease [26,27] with the *adeA* gene at a high targeting efficiency (unpublished). These results indicate that the *adeA* gene is an effective marker for gene disruption and aids in the experimental progress of *A. oryzae* genetics.

In continuation of the present study on developing a novel transformation system with quadruple auxotrophy in *A. oryzae*, it may be possible to conduct multiple gene disruptions for breeding of industrially favored strains. As mentioned earlier, disruption of some important protease encoding genes may lead to a higher yield in heterologous protein production [28]. However, as yet studies on disruption of more than two genes have not been reported in *A. oryzae*. Towards an effort in this direction, we have recently succeeded in the disruption of *A. oryzae pepA* gene, which in combination with the manipulation of other proteases could be a favorable strategy for efficient production of heterologous proteins in *A. oryzae*. The lack of sexual life cycle in *A. oryzae* makes it impossible to increase the number of auxotrophic transformation markers by sexual crossing. In this study following the screening for adenine auxotrophy by red colony-color selection [15], we bred quadruple auxotrophic hosts from the triple one by disruption of the *argB* gene with the *adeA* gene and another step of red-color colony selection. This approach constitutes rapid screening and gene disruption methodology with a high frequency, thus providing a very efficient way for the isolation of multiple auxotrophic mutants in *A. oryzae* industrial strains in which transformation system has not yet been developed. While repetitive rounds of selection of *ade* minus mutants in the present study may lead to the strain acquiring multiple copies of *ade* genes, it is possible to improve this method by adopting blaster strategies previously reported in repeated use of the *pyrG* gene as a selection marker in *Aspergillus fumigatus* [29] and *ura*-blasters in *Saccharomyces cerevisiae* and *Candida albicans* [30,31]. This strategy can also be a powerful tool for developing transformation systems with multiple auxotrophy in other deuteromycetes which find wide usage in industry and agriculture.

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