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Integrative Transformation of *Aspergillus oryzae* with a Plasmid Containing the *Aspergillus nidulans argB* Gene

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The transformation of *Aspergillus oryzae* has been achieved with a plasmid carrying the *Aspergillus nidulans argB* gene coding for ornithine carbamoyltransferase (OCTase). The frequency of transformation was relatively low (0.7 transformants/μg DNA) but the transformed phenotype was extremely stable for many generations without selective pressure.

Southern blot analysis revealed that transformation had occurred by integration of multiple tandem copies of plasmid DNA into the host genome through non-homologous recombination. There was no evidence of the existence of free plasmid in the transformants. The number of integrated copies of the plasmid ranged from 15 to 60. The specific activity of OCTase in the cell-free extract was proportional to the copy number of the plasmid, indicating that most of the integrated *argB* gene was expressed.

Aspergillus oryzae has been important in fermentation industries in Japan, *i.e.*, sake, shoyu and miso manufacture as well as the production of industrial enzymes. Strain improvement in *A. oryzae* has been carried out by mutation and protoplast fusion,[1] but not yet by genetic transformation, since vectors carrying selectable markers for the development of a transformation system are not readily available.

In the previous paper,[2] we reported that a hybrid plasmid containing a genomic DNA fragment of *A. oryzae* was able to complement a methionine-auxotrophic mutation of this organism. The transforming plasmid, which was recovered from *Aspergillus* by transformation into *Escherichia coli*, appeared to exist in the transformant as a self-replicating circular DNA. These findings facilitated the construction of efficient transformation systems and plasmid vectors carrying selectable genetic markers for *A. oryzae*. However, since it has not been determined what kind of enzyme is encoded by the isolated DNA fragment, genetic and biochemical studies on the transformants have not been performed.

On the other hand, for *Aspergillus nidulans*, a closely related species to *A. oryzae*, genetic studies and transformation systems have been well developed. At present three different selectable marker genes of *A. nidulans* are mainly used for transformation experiments with this organism.[3] In particular, of the available genes, the *argB* gene[4] coding for ornithine carbamoyltransferase (OCTase; EC 2.1.3.3) contains no introns[5] and allows the complementation of the arginine-auxotrophic mutation of *E. coli*,[6] *Saccharomyces cerevisiae*,[7] *Neurospora crassa*[8] and *A. niger*[9] as well as *A. nidulans*.[10～13]

We report here the successful transformation of an *A. oryzae* arginine-auxotrophic mutant, using a plasmid which contained the *argB* gene of *A. nidulans*. Molecular analysis revealed that multiple tandem copies of the input plasmid were integrated into the host genome. Moreover, the high level of OCTase activity suggested that most of the integrated *argB* genes were expressed. These results will open the way to the expression of foreign genes coding for useful enzymes in *A. oryzae*.

MATERIALS AND METHODS

Strains and media. *A. oryzae* FN-16 isolated by Hara *et al.*[14] was used as the wild-type strain. Recipient strains deficient in OCTase were obtained by UV irradiation of conidia of FN-16. One of them, M-2-3, which was used in transformation experiments, formed white conidia and could grow on the minimal medium (MM) supplemented with citrulline or arginine but not with ornithine. *A. nidulans* IAM 2006 was also used as a standard strain containing the *argB* gene. *E. coli* JA221 (*hsd*R *lac*Y *leu*B6 *trp*E5 *rec*A1) was used for plasmid preparation and for rescuing the transforming plasmids from *A. oryzae*.

The media for the growth of *Aspergillus* were as follows; Czapek-Dox as the minimal medium (MM), *koji*-extract as the complete medium (CM) and dextrin-pepton (DP), used for mycelial preparations, all being as described previously.[2]

Plasmids. Plasmid pSa143[7] used for transformation was kindly provided by Prof. P. Weglenski. It carries a 2.6kb fragment of *A. nidulans* DNA containing the whole *argB* gene. pSa123 was obtained from pSa143 by removal of two small *Eco*RI fragments containing yeast *LEU2* and 2 μm DNA. Plasmid pRBM1 was constructed by inserting the 3.5 kb *Bam*HI fragment, which contains the gene of *A. oryzae* complementing the methionine-auxotrophic mutation, from pKA5-1[2] into pSa123. It was used as a probe for determination of the copy number of transforming plasmids integrated into the host genome (Fig. 1).

DNA preparations. Plasmids were isolated from large scale cultures of *E. coli* by the alkaline lysis method[15] and then purified by CsCl/EtBr centrifugation. Total cellular DNA of *Aspergillus* was prepared from protoplasts as described previously.[2]

Transformation experiments. Transformation of *E. coli* was carried out according to the standard method.[16] Transformation of *A. oryzae* was performed by the method described[2] with slight modifications. The protoplasts were suspended in 5% NaCl, 10 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5) at the concentration of 2.5×10^8/ml, and then divided into 0.2 ml aliquots. Plasmid DNA (10 μl in TE) was added and then each mixture was placed on ice for 30 min. Then 1 ml of 25% (w/v) PEG4000, 50 mM $CaCl_2$, 10mM Tris–HCl (pH 7.5) was added with gentle mixing, followed by incubation for 15 min at room temperature. The suspensions were diluted with 6 ml of 5%NaCl, 10 mM $CaCl_2$, 10 mM Tris–HCl (pH7.5) and then centrifuged ($700 \times g$, 5 min). After washing with the same buffer, each protoplast suspension was placed on an MM agar plate containing 5% NaCl and then overlayed with soft agar (0.5% agar, 42°C). The plate was incubated at 30°C for 3~10 days.

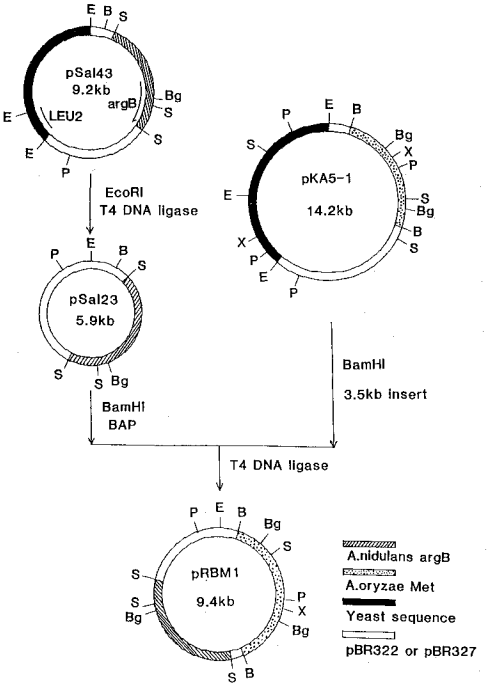

FIG. 1. Restriction Map of pSa143 and Construction of pRBM1.

Restriction enzymes: *B*, *Bam*HI: Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

Hybridization analysis. The DNA fragments digested with appropriate restriction enzymes were separated on a 0.8% agarose gel and then transferred to a nylon membrane (Hybond N, Amersham) by the method of Southern.[17] For dot blotting, total cellular DNA was denatured at 100°C for 5 min and then spotted on a nylon membrane following serial doubling dilutions. DNA probes were labeled with α-^{32}P-dCTP by nick translation.[18] Hybridizations were performed in 5 × SSC, 5 × Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 50% (v/v) deionized formamide at 42°C for 20 hr. The hybridized membranes were washed for 1 hr each with 2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS at 65°C.

Enzyme assays. Mycelia of wild type *A. oryzae*, transformants and *A. nidulans* grown in liquid MM and CM were suspended in 5 volumes of 10 mM phosphate buffer (pH 6.0) and then ground with sea sand in a cold mortar. After centrifugation ($20{,}000 \times g$, 15 min, 4°C), the supernatant was retained as the cell-free extract. OCTase was assayed as the formation of L-citrulline. The reaction mixture, containing 0.7 ml 0.1 M Tris–HCl (pH 8.0), 0.1 ml 50 mM L-ornithine and 0.1 ml cell-free extract suitably diluted, was incubated at 37°C for 5 min. The reaction was initiated by the addition of 0.1 ml of freshly prepared 50 mM carbamyl

phosphate. After 10 min incubation at 37°C, 1 ml of 15% perchloric acid was added to stop the enzyme reaction. L-Citrulline formed was determined by the colorimetric method of Prescott and Jones.[19] The amount of protein in the extract was determined by Lowry's method.[20]

RESULTS

Transformation of an A. oryzae arginine-auxotrophic mutant with pSa143

Strain M-2-3 was used as the recipient in the transformation experiment. No colonies were detected when conidia of the strain (2×10^8) were plated on MM. Approximately 5×10^7 protoplasts were mixed with 10 μg of pSa143 DNA in the presence of PEG4000 and $CaCl_2$, and then regenerated in soft agar overlays. The regeneration frequency of the protoplasts on a CM plate before and after PEG addition, as compared to the initial number of protoplasts determined microscopically, was 50 ~ 70% and 5 ~ 7%, respectively. The latter was due to the aggregation of protoplasts followed by fusion after PEG addition. On MM plates a few large colonies were observed together with several small ones. No colonies appeared on MM in the experiment with no plasmid or pBR322. Most of the small colonies showed stunted growth and were thought to be "abortive" transformants, as reported by Tilburn *et al.*[21] A similar observation was made on the transformation of *A. oryzae* with pKA5-1, as described previously.[2]

Counting the large colonies as real transformants, the frequency was 0.7 transformants per μg of plasmid DNA. The frequency in this study is much lower than that obtained for *A. nidulans*[10~13,21~24] but approximately the same as that in the transformation experiment with *A. niger* using a plasmid containing *A. nidulans argB* gene.[9]

Three of the 7 transformants, showing vigorous growth on successive transfers to MM, were selected for detailed analysis.

Mitotic stability of the transformants

The 3 transformants were grown on CM agar at 30°C until conidiation. The conidia formed were harvested in 0.01% Tween 80 and then plated on a CM plate following appropriate dilutions. The resultant single colonies were transferred onto MM to determine the number of prototrophs. As shown in Table I, all of the transformants were sufficiently stable during vegetative growth on CM. In particular, no auxotrophs were observed for transformant TR2. The high stability of the transformed phenotype suggested that the transforming plasmid was integrated into the host genome.

TABLE I. MITOTIC STABILITY OF THE TRANSFORMANTS

Transformant	Colonies counted		Stability (%)
	CM	MM	
TR1	118	110	93
TR2	125	125	100
TR3	106	88	83

The transformants were incubated on CM slants until conidiation. The conidia formed were plated on CM and the resulting colonies were then transferred onto MM plates to determine the mitotic stability.

Transformants TR1 and TR3 were relatively unstable compared to TR2. This can be explained by heterokaryosis of the transformants rather than by loss of expression following excision of the integrated plasmid from the chromosome. Since *A. oryzae* conidia are multinucleate (generally 4 nuclei per conidium[25]), the resulting transformants are heterokaryotic, in which nuclei containing the integrated plasmid and ones not having such sequences exist. During mitotic divisions, TR1 and TR3 in the heterokaryotic state formed a few conidia consisting of only untransformed nuclei, while TR2 was apparently homokaryotic and gave no arginine-auxotrophic segregants. Once TR1 and TR3 have become homokaryotic on further purification, their transformed phenotypes are extremely stable for many generations.

Plasmid rescue from the transformants

Although it was suggested, as described above, that the transformation of *A. oryzae* was due to integration of the plasmid, the

TABLE II. PLASMID RESCUE FROM THE TRANSFORMANTS

DNA source	No. of amp^R transformants		
	Uncut	*Bam*HI	*Eco*RI
M-2-3 (recipient)	0	0	0
TR1	0	0	6
TR2	0	52	143
TR3	0	3	5

50 μg of DNA from each of the transformants was digested with restriction enzymes followed by ligation, and then used for the transformation of *E. coli* JA 221.

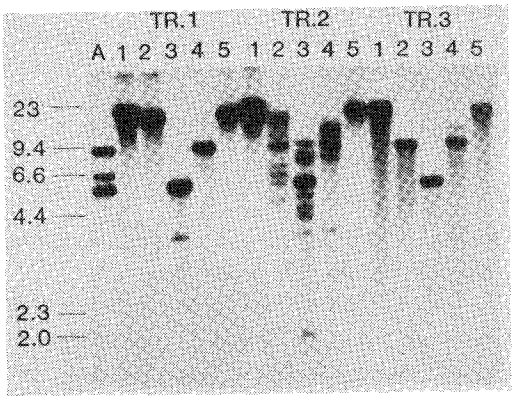

FIG. 2. Southern Blot Analysis of Transformant DNA. Transformant DNAs (10 μg) undigested (lane 1) and digested with *Bam*HI (lane 2), *Eco*RI (lane 3), *Bgl* II (lane 4) and *Xho*I (lane 5) were electrophoresed on 0.8% agarose, blotted and then probed with ^{32}P-labeled pBR322. Lane A contains pSa143 fragments digested with *Bam*HI (9.2 kb), *Sal* I (6.6 kb) and *Eco*RI (5.9 kb). The sizes of molecular standards (*Hind*III digests of λ phage DNA) are given in kb.

possibility of the existence of free plasmid in the transformants could not be ruled out. To examine this possibility, total cellular DNA recovered from the transformants was used for the transformation of *E. coli* to ampicillin resistance. As shown in Table II, no transformants of *E. coli* having ampicillin resistance were obtained in the experiments with undigested DNA. On the other hand, DNA digested with *Bam*HI or *Eco*RI followed by ligation was able to transform *E. coli*. These results also indicate that the transforming plasmid was integrated into genomic DNA, and did not exist as a free circular plasmid. The difference in the transformation efficiency of *E. coli* with digested DNA from the transformants will be discussed below.

Molecular analysis of the transformants

To examine the state of the transforming plasmid, total cellular DNA was subjected to hybridization analysis. Figure 2 shows the results of hybridization with pBR322 of transformant DNAs before and after digestion with four restriction endonucleases. In the undigested DNA lane, only one hybridization band was found in the region of chromosomal DNA, whereas no hybridization to lower molecular weight DNA could be detected even on long exposure, indicating that the DNA sequence corresponding to pBR322 was integrated into the chromosome and that no free plasmids were present in the transformants. DNA from the wild type and recipients had no homologous sequences to pBR322, as expected (data not shown). In addition, when DNA of the wild type or a recipient was probed with pSa143 under less stringent conditions (37°C, 50% formamide, with washing in 2×SSC, 0.1% SDS, at 42°C), no bands hybridizing to the probe were detected (data not shown), indicating that sequences strongly homologous to *A. nidulans argB*, yeast *LEU2* and 2 μm DNA are absent in *A. oryzae* genomic DNA.

Only the hybridization of DNA digested with *Xho*I, which does not cut within pSa143, to high molecular weight DNA was observed. When digested with *Bam*HI or *Bgl*II, which cut once in the plasmid, a strong hybridization band at 9.2 kb length of the plasmid was observed except for the *Bam*HI digest of TR1 DNA. These results show that multiple tandem copies of the plasmid were integrated into the host genome. The few additional minor bands evident in DNA digests of TR1 and TR3 can be explained as modifications due to internal rearrangement. For TR2 a number of additional bands were observed, indicating that integration at two or more sites had occurred besides rearrangement. This is consistent with the high transformation ef-

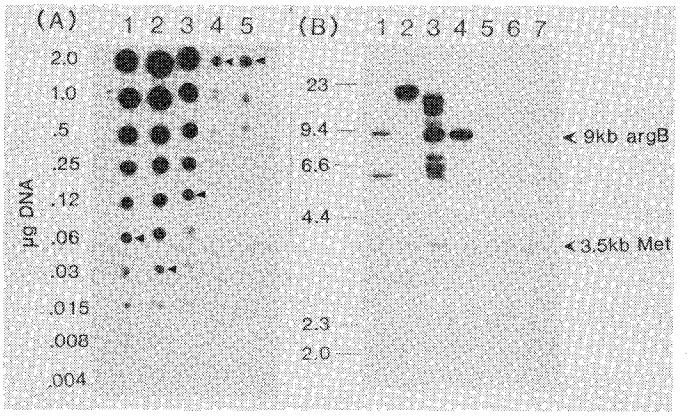

FIG. 3. Determination of the Copy Number of the Plasmid.

(A): Total cellular DNA was serially diluted two-fold, from 2 μg, and then spotted on a nylon membrane, followed by hybridization with ^{32}P-labeled pRBM1. The spots showing the same intensities are indicated by arrows. Lanes: 1, TR1; 2, TR2; 3, TR3; 4, M-2-3; 5, *A. nidulans* IAM 2006.

(B): *Bam*HI digests of DNA were electrophoresed on 0.8% agarose, blotted and then probed with ^{32}P-labeled pRBM1. Lanes: 1, pSa143 fragments digested with *Bam*HI and *Eco*RI; 2, TR1; 3, TR2; 4, TR3; 5, M-2-3; 6, FN-16; 7, *A. nidulans* IAM 2006. The 3.5 kb band in lanes 2~6 and the 9 kb band in lane 7 correspond to the chromosomal Met gene of *A. oryzae* and the chromosomal *argB* of *A. nidulans*, respectively. The sizes of molecular standards are given in kb.

TABLE III. SPECIFIC ACTIVITY OF OCTASE IN THE TRANSFORMANTS

Strain	Czapek-Dox medium		Dextrin-pepton medium	
	Spec. act.[a]	Ratio[b]	Spec. act.[a]	Ratio[b]
FN-16	17.1	1.0	2.8	1.0
M-2-3	—	—	0.6	0.2
TR1	469.9	27.5	71.3	25.6
TR2	904.6	52.9	133.7	47.9
TR3	514.3	30.1	42.3	15.2
2006[c]	22.2	1.3	3.4	1.2

[a] Specific activity; μg L-citrulline/min/mg protein.
[b] Specific activity of FN-16 is expressed as 1.0.
[c] *Aspergillus nidulans* IAM 2006.

ficiency of *E. coli* by TR2 DNA, which was digested with *Bam*HI or *Eco*RI followed by ligation as described above. The high molecular weight DNA in the *Bam*HI digest of TR1 DNA hybridizing to pBR322 can be explained by disruption of the *Bam*HI site in the plasmid during or after integration.

Determination of the copy number of the integrated plasmid

The number of integrated copies was determined by dot blot analysis. As a probe, pRBM1 containing *A. nidulans argB* and the *A. oryzae* 3.5kb fragment (Met) was used, which is taken as one copy in *A. nidulans* and *A. oryzae*, respectively. By comparing the amounts of DNA from the transformants giving the same intensity of hybridization as 2 μg of DNA from the recipient or *A. nidulans* wild type (Fig. 3(A)), the copy number of the integrated plasmid in TR1, TR2 and TR3 could be estimated to be at least 30, 60 and 15, respectively. The Southern hybridization of *Bam*HI digests followed by densitometry

shown in Fig. 3(B) also indicated similar copy numbers.

Expression of OCTase in the transformants

To determine whether or not multiple copies of the *argB* gene were expressed in the transformants, the activity of OCTase in cell-free extracts was assayed. As shown in Table III, the specific activity of the enzyme in the transformants was much higher than that in wild type *A. oryzae* and *A. nidulans*. In addition, a higher level of OCTase activity reflected the copy number in the transformant. As expected, there were no differences in other enzyme activities, such as that of arginase, among the transformants and wild type *Aspergillus* (data not shown). This shows that the transformants expressed most of the multiple copies of the *argB* gene. Further studies on *argB* gene expression at transcriptional and translational levels will be needed. Since pSa143 carries yeast *LEU2* coding for β-isopropylmalate dehydrogenase in addition to the *argB* gene, expression of the yeast gene in *A. oryzae* is also of interest.

DISCUSSION

The successful transformation of an *A. oryzae* arginine-auxotrophic mutant with a plasmid carrying the *A. nidulans argB* gene described here allows the use of this heterologous gene as a selectable marker for a transformation system in *A. oryzae* as well as in other filamentous fungi.[6] In this study, the transforming DNA was integrated into the host genome and no evidence could be obtained for the existence of free plasmid in the transformants. Thus pSa143 used for transformation contains no sequences which function as autonomous replicators in *A. oryzae*.

In *A. oryzae* chromosomal DNA there are no sequences with extensive homology to plasmid pSa143, indicating that integration into the genome has occurred at non-homologous sites. This agrees with the results of transformation experiments on *A. nidulans*[13,26] and *A. niger*,[9,27] but differs from those with the yeast, *S. cerevisiae*, for which integration events were found to be strongly dependent on homology.[28] Nevertheless, it cannot be excluded that homologous sequences are required for efficient integration into the genome, resulting in a higher frequency of transformation than that obtained in this study. Therefore, it is necessary for improvement of the transformation frequency to construct a hybrid plasmid, such as pRBM1, by the addition of DNA sequences homologous to the host genome.

Southern hybridization analysis of the transformant DNA digested with several restriction enzymes revealed that multiple copies of the plasmid were integrated into the genome in tandem arrays. The copy number of the integrated plasmid determined by dot blot analysis ranged from 15(TR3) to 60(TR2). Similar results were obtained in transformation experiments on other *Aspergillus* species.[9,13,22,23,27] Models have been proposed to explain the integration of the tandem repeats into the genome[9,23]: (1) the transforming plasmid forms a multimer prior to integration, or (2) successive homologous recombinations into the integrated plasmid take place following insertion of a single copy. These hypotheses indicate the possibility that a pre-existing multimeric plasmid is integrated into the genome repeatedly by homologous recombination resulting in the formation of more than 30 tandem arrays with a length of about 300 kb, as found in transformant TR1. In all the transformants investigated in this study, tandem integration of mutiple copies of the plasmid had occurred, which was ascribed to the specificity of the recipient strain, as reported by Wernars *et al.*[23]

Enzyme assays showed that the specific activity of OCTase was almost proportional to the number of the integrated copies in the transformants, indicating that high levels of expression of foreign genes in *A. oryzae* will be possible. Moreover, the extremely high stability of the transformed phenotype was observed even on non-selective medium for many generations, allowing the cultivation of the transformant

cells in natural media, which is advantageous as to the economy of industrial production.

The results in this study indicate that the newly developed transformation system for *A. oryzae* will allow the integration of multiple copies of desired genes into a host genome with stable maintenance, and will lead to high yields of gene products.

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