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The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: A convenient and general selection system for filamentous fungal transformation

Shiela E. Unkles[1], Edward I. Campbell[1], Yolanda M.J.T. de Ruiter-Jacobs[2], Martien Broekhuijsen[2], Janet A. Macro[1], Dirk Carrez[3], Roland Contreras[3], Cees A.M.J.J. van den Hondel[2], and James R. Kinghorn[1,*]

[1] Plant Molecular Genetics Unit, Sir Harold Mitchell Building, University of St. Andrews, St. Andrews, Fife KY16, Scotland
[2] TNO Medical Biological Laboratory, PO. Box 45, NL-2280AA Rijswijk, The Netherlands
[3] Laboratorium voor Molculaire Biologie, K.L. Ledeganckstraat 35, State University of Gent, B-9000 Gent, Belgium

Summary. The development of an efficient and homologous transformation system for *Aspergillus oryzae* is described. This is based on nitrate reductase (*nia*D) of the nitrate assimilation pathway. The *nia*D system offers a number of inherent advantages over many other systems and may be of general use for nitrate-utilising filamentous fungi. Transformation frequencies of up to 800 transformants per microgram DNA are observed with *A. oryzae*. The preponderance of integration events take place at the resident *nia*D locus either by gene conversion (41%), single integration (23%) or multiple tandem integration (36%). Heterologous expression of the *A. oryzae nia*D gene in the filamentous fungi *A. nidulans*, *A. niger* and *Penicillium chrysogenum* is observed. That heterologous putative *nia*D hybridisation signals are seen with other fungal DNAs affords the opportunity to isolate the corresponding *nia*D from various fungi in order to develop homolgous transformation. Co-transformation with the introduction of the non-selected markers *pyr*G, *tub*-2, and *uid*A has been achieved.

Key words: *Aspergillus oryzae* – Homologous transformation – Nitrate reductase – Chlorate resistance – Co-transformation

Introduction

The filamentous fungus *Aspergillus oryzae* is an important industrial organism for the commercial production of oriental fermented foods (Hesseltine 1983) including miso, saké and soya sauce, as well as extracellular enzymes such as α-amylase and proteases. The food fermentation industry is considerable and, although mainly based in the Orient, such processes are beginning to be carried out in the Western Hemisphere. The commercial production of extracellular enzymes is enormous in the West. Indeed, the first process describing the production of *A. oryzae* enzymes for starch hydrolysis was patented in 1894. Since *A. oryzae* secretes proteins at high levels, its future potential as a host for the secretion of foreign protein, including mammalian proteins is evident. Moreover, the World Health Organisation regards *A. oryzae* as one of the few filamentous fungi which is acceptable, from a health standpoint, for such commercial purposes (World Health Organisation Report 1987).

The availability of molecular genetic technology offers the possibility of new routes to strain improvement. Recently gene mediated transformation systems (a pre-requisite for recombinant DNA techniques) have been developed for *A. oryzae*. These include *A. niger pyrG* (Mattern et al. 1987), *A. nidulans arg*B (Hahm and Batt 1988), as well as *A. oryzae met* (Iimura et al. 1987). The frequencies of transformation in these systems are relatively low (5–20 transformants per microgram DNA) compared with transformation systems in the related fungus *A. nidulans* (Ballance and Turner 1985; Hynes 1986; Johnstone 1985; Tilburn et al. 1983; Yelton et al. 1984).

We have attempted to assess the suitability of the nitrate assimilation pathway for the development of transformation systems in filamentous fungi. This system offers many inherent advantages. First, mutants may be easily obtained by positive selection via resistance to chlorate. Second, that this can be done by spontaneous means, reduces the possibility of secondary mutations arising in genes of commercial interest or in genes encoding essential catalytic steps. Third, the desired mutants have a simple growth phenotype, i.e. inability to utilise nitrate as sole nitrogen source (see below). Fourth, the pathway is dispensable and therefore mutations in the nitrate pathway should not alter growth or metabolic fluxes through important pathways. Fifth, most filamentous fungi will utilise nitrate as a sole nitrogen source.

The catalytic steps and genes (for reviews see Arst 1984; Cove 1979; Arst and Scazzocchio 1989; Kinghorn 1989) involved in the related fungus *A. nidulans* and many other fungi (for a review see Unkles 1989) are shown in Fig. 1. Mutants defective in the structural gene *nia*D for the nitrate reductase apoprotein (EC 1.6.6.8) but not nitrite reductase (*nii*A) can be obtained on the basis of their resistance to chlorate. In addition mutations resulting in chlorate resistance can occur in a series of genes, such as the transport gene *crn*A and genes *cnx*A–J required for the biosynthesis of a molybdenum co-factor which is necessary for nitrate

Offprint requests to: J. Kinghorn

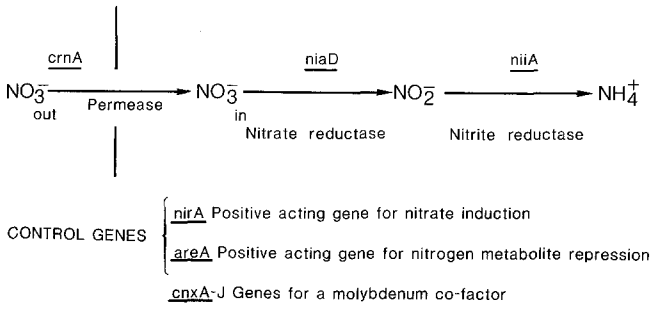

Fig. 1. The catalytic conversion of nitrate to ammonium. Extracellular nitrate is transported into the cell by a permease encoded by the *crn*A gene, converted to nitrite by the action of nitrate reductase encoded by *nia*D, and finally converted to ammonium by nitrite reductase, the *nii*A gene product. The *nir*A, *are*A and *cnx*A genes are discussed in the text

reductase as well as xanthine dehydrogenase activity. Chlorate resistant mutants can also be obtained as a result of mutation within *nir*A and *are*A, positive-acting control genes for the system. Fortuitously, *nia*D mutants can be differentiated from *crn*A, *cnx*A–J, *nir*A, and *are*A mutants on the basis of simple growth tests.

We describe here the isolation of *nia*D mutants, the isolation of the *A. oryzae nia*D gene and the suitability of the system for transformation.

Materials and methods

Strains, media and plasmids. Escherichia coli strain DH5 or JM109 was used for construction, propagation and amplification of hybrid plasmids (Frischauf et al. 1983; Maniatis et al. 1982). The *E. coli* strains NM538 and NM539 were used for construction (Frischauf et al. 1983; Maniatis et al. 1982) of the *A. oryzae* gene library. The *A. oryzae* wild-type strain IMI144242 (=ATCC91002, α-amylase over-producing strain) was used for selection of recipient mutants. *A. oryzae* strain A04.1 which is an *nia*D14 *pyr*G1 double mutant derived from IMI-144242 (Mattern et al. 1987), was used for *pyr*G co-transformation experiments. The *A. nidulans nia*D10 deletion mutant strain used for heterologous transformation experiments was that isolated and described by Tomsett and Cove (1979). Standard media for cultivation of bacteria and fungi were as described in Maniatis et al. (1982) and Cove (1966), respectively. Plasmid pSTA8 containing the *A. nidulans nia*D gene (Johnstone et al. 1989) was used as source for probes for the *A. oryzae* gene equivalent. Plasmid vector pNOM102 containing the *A. nidulans gpd-E. coli uid*A fusion gene was constructed by Roberts et al. (1989). pBT3 containing the *Neurospora crassa tub*-2 gene which confers benomyl resistance was reported by Orbach et al. (1986). pA04-2 harbouring the *A. oryzae pyr*G gene was isolated by de Ruiter-Jacobs et al. (1989).

Isolation of niaD *mutants.* Nitrate assimilation defective spontaneous mutants were obtained by positive selection for resistance to chlorate (Cove 1979). Spores ($\sim 10^9$) were harvested in 10 ml saline containing 0.08% Tween 80. Aliquots (0.2 ml) were spread out on minimal medium containing 470 mM chlorate with 10 mM glutamate as the sole source of nitrogen.

DNA isolation and manipulation. Isolation of chromosomal DNA from wild-type *A. oryzae* and transformants was by the method of Tilburn et al. (1983). Standard recombinant DNA techniques were used for the cloning and characterisation of the *nia*D gene and for analysis of transformants (Johnstone et al. 1989). Construction and amplification of the gene bank of *A. oryzae* wild-type strain (IMI44242) was as described previously (Van Hartingsveldt et al. 1987). A primary library of 98000 plaques, representing 40–50 copies of the genome, was obtained.

DNA hybridisation conditions. Following conventional Southern blotting (Maniatis et al. 1982) on to nylon membranes (Hybond-N, Amersham), hybridisations were performed in plastic sandwich boxes containing 2–3 ml prehybridisation/hybridisation solution per square centimetre. This solution contained 5 × SSPE, 6% PEG 6000 (BDH Chemicals, Poole, UK) 0.5% skimmed milk (Marvel, Cadbury, UK) 0.1% $Na_4P_2O_7$, 1% SDS and 250 μg/ml sheared, boiled herring sperm DNA. After prehybridisation for 4–5 h, probe was added and hybridisation continued overnight. For low or high stringency, hybridisation and washing temperatures were 54° or 65° C respectively. Membranes were washed in 5 × SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ for low stringency or 0.2 × SSC, 0.1% SDS, 0.1% $Na_4P_2O_7$ for high stringency.

Protoplast preparation and transformation. A. oryzae mycelial cells grown at 30° C for 12 h were collected and resuspended in lysis buffer (0.8 M $MgSO_4$, 10 mM PO_4, pH 5.8). Novozyme 234 (batch number PPM2715; Novo Biolabs, Denmark) was added to a concentration of 5 mg/ml, the mixture incubated at 30° C for 45 min with gentle shaking and centrifuged at 3000 rpm for 10 min. Four volumes of 1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM TRIS, pH 7.5 was added to the supernatant and the solution centrifuged at 2000 rpm for 5 min. Pelleted protoplasts were washed 3 times in 1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM TRIS, pH 7.5 and finally resuspended in the above buffer at a concentration of $1–2 \times 10^7$ protoplasts in 100 μl. To this suspension, 1–5 μg plasmid DNA was added together with 12.5 μl 50% w/v PEG 4000, 50 mM $CaCl_2$, 10 mM TRIS, pH 7.5 and allowed to stand on ice for 20 min. This suspension was plunged into 1 ml of the PEG solution, mixed for a couple of seconds before diluting out the effect of the PEG with 2 ml of the sorbitol solution. The suspension was added to transformation selection medium (minimal medium containing 1.2 M sorbitol and 10 mM nitrate as sole nitrogen source) cooled to 45° C. The plates were incubated at 30° C for up to 5 days. Control protoplasts were treated as above but without addition of DNA. Protoplast viability was tested by suspending protoplasts in non-selective medium. Viability was usually about 10%.

Results

Generation of niaD *mutants*

It was found that spontaneous chlorate resistant *A. oryzae* mutants arose at a frequency of 1 in 10^6 viable spores. In all 73 mutant chlorate resistant strains were isolated and further purified on chlorate-containing minimal medium with glutamate as the sole source of nitrogen. The ability of these mutants to grow on nitrate, nitrite, ammonium,

Table 1. Growth tests of *Aspergillus oryzae* chlorate resistant mutant strains on various nitrogen sources

Nitrogen source[a]					Putative[b] mutation	Observed number
Nitrate	Nitrite	Hypoxanthine	Glutamate	Ammonium		
−[c]	+	+	+	+	*nia*D	11
+	+	+	+	+	*crn*A	6
−	+	−	+	+	*cnx*	28
−	−	+	+	+	*nir*A	28
−	−	−	−	+	*are*A	0

[a] Nitrogen sources at a concentration of 10 mM
[b] Denotes loss-of-function mutations
[c] + denotes wild-type growth whilst − denotes no growth

hypoxanthine, proline or glutamate as sole nitrogen source was assessed. Eleven had a phenotype indicative of nitrate reductase structural mutants (*nia*D), i.e. they failed to grow with nitrate but grew on the other nitrogen sources (Table 1). It was found on testing three putative *nia*D mutants for reversion to nitrate prototrophy, that one allele, namely *nia*D14, showed a reversion frequency of less than 1 in 10^7 viable spores and hence this strain, designated A01.1, was considered to be a suitable stable recipient for transformation experiments. Strain A01.1 was used to make an *nia*D14 *pyr*G1 double mutant (designated strain A04.1) for co-transformation experiments with *pyr*G (Mattern et al. 1987).

Non-reverting *nia*D mutants of *A. niger* (derived from wild-type strain ATCC10864) and *Penicillium chrysogenum* (from wild-type strain V899) were isolated using the same approach. [Mutants in *nia*D of *P. chrysogenum* industrial strains have been reported previously by Birkett and Rowlands (1981) but were not made available.] Chlorate resistant mutation frequencies were observed for *A. niger* and *P. chrysogenum* similar to that for *A. oryzae* as was the frequency of *nia*D, *cnx* and *nir*A growth phenotypes.

Isolation and characterisation of the A. oryzae niaD *gene*

A 2.3 kb *Xba*I fragment containing the *A. nidulans nia*D nitrate reductase structural gene was hybridised against various genomic DNAs digested with *Eco*RI. Unique bands of 2.8, 2.5 and 6 kb were observed with *A. niger*, *A. oryzae* and *P. chrysogenum* respectively (Fig. 2). From an *A. oryzae* library of 40000 plaques hybridised with the *A. nidulans* 2.3 kb *Xba*I fragment 17 positive clones were identified. Two of these (λSTA51 and λSTA62) were purified further. Both contained *Sal*I fragments (5.5 kb and 8.2 kb respectively) which hybridised strongly to the *A. nidulans nia*D probe and these overlapping fragments were subcloned into pUC18 to give plasmids pSTA13 and pSTA14 respectively. Only pSTA14 (Fig. 3) was capable of phenotypic rescue of the *A. oryzae nia*D14 mutant to nitrate utilisation and so it was assumed that pSTA14, but not pSTA13, did in fact contain the entire structural *nia*D gene. Radiolabelled *Xba*I-digested pSTA14 was hybridised to a Southern blot of wild-type *A. oryzae* DNA cut with enzymes which cleave the recombinant clone. Identical bands were observed using either pSTA14 or the *Xba*I fragment of *A. nidulans nia*D (data not shown). This showed positive identification of the clone. No evidence for rearrangements of the clone sequences in pSTA14 (by comparison with wild-type genomic

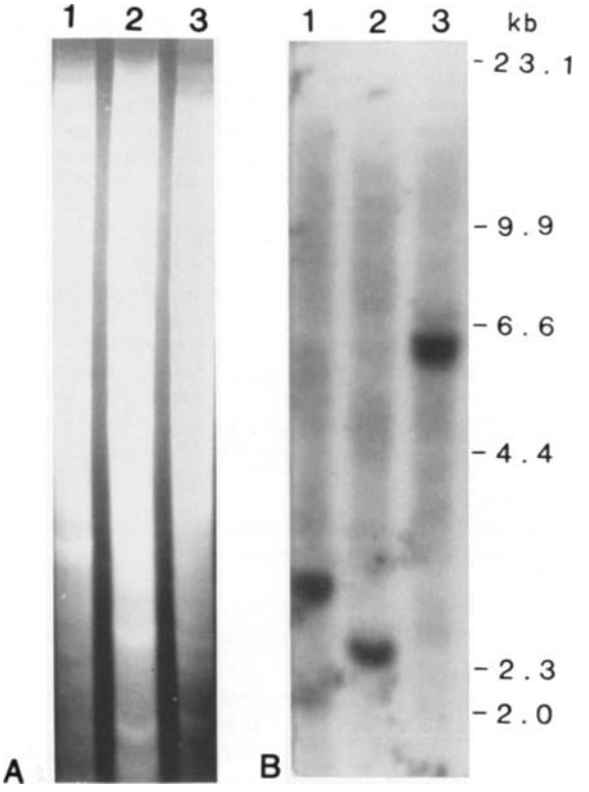

Fig. 2A and B. Heterologous hybridisation of the *Aspergillus nidulans nia*D gene. **A** Genomic DNA from (1) *A. niger*, (2) *A. oryzae* and (3) *Penicillium chrysogenum* was digested with *Eco*RI and fragments separated on 0.8% agarose. **B** Following transfer to nylon, the blot was probed with a 2.3 kb ^{32}P-labelled *Xba*I fragment containing the *A. nidulans nia*D gene. Hybridisation was at low stringency (54° C, washed once with 5 × SSC). The positions of λ*Hin*dIII molecular size markers are indicated

sequences) was detected (Fig. 4). In this regard the expected internal fragments, 2.5 and 1.8 kb *Bam*HI, 2.5 kb *Eco*RI and 4.2 kb *Bgl*II, were observed.

Homologous transformation

Transformation of *A. oryzae nia*D14 with circular pSTA14 gave an average of 64 transformants per microgram DNA (Table 2). The background growth with nitrate as sole nitrogen source, was extremely poor and no abortive transfor-

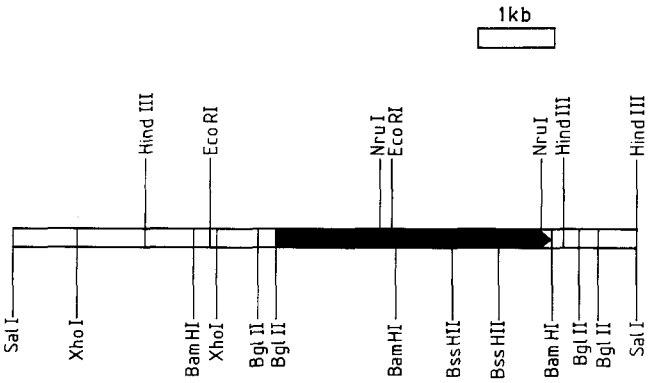

Fig. 3. Restriction map of pSTA14 containing the *Aspergillus oryzae* nitrate reductase. The map was determined by single and double digestions using the enzymes indicated. Vector sequences are not included. No sites were found for enzymes *Bsc*I, *Pst*I, *Sma*I, *Xba*I, or *Xma*I. The approximate position of the *nia*D gene within pSTA14 is indicated by the *solid bar*. The start of the *nia*D gene was determined by hybridisation to an extreme 5′ *A. nidulans nia*D fragment. The *arrow* indicates the direction of transcription as judged by hybridisation to a 3′ *A. nidulans nia*D probe (data not shown). Hybridisation experiments were carried out at 54° C followed by washing in 5 × SSC

Table 2. Transformation of *Aspergillus oryzae nia*D14 with the homologous *nia*D gene[a]

Plasmid	Form	Frequencies[b]	Average
pSTA14	Circular[c]	64, 77, 51, 67	64
pSTA14	Linear[d]	236, 406, 386, 789	455

[a] *A. oryzae* strain A01.1 (*nia*D14)
[b] Number of transformants per microgram DNA. These represent results from four independent experiments
[c] Uncut plasmid
[d] Plasmid digested with *Hin*dIII

mants were observed (Fig. 5). Some transformants initially grew slower than the others but did in fact catch up and develop into mature colonies. Transformation levels increased to an average of 455 transformants per microgram DNA when pSTA14 was cleaved with *Hin*dIII (Fig. 3). Other fungal research groups have likewise observed increases in fungal transformation frequencies using linearised plasmid molecules depending on the position of the site of cleavage (Dhawale and Marzluf 1985; Skatrud et al. 1987; Wang et al. 1988).

To determine the types of integration events that took place using uncut pSTA14, chromosomal DNA of 22 nitrate-utilising transformants was analysed by Southern blotting followed by hybridisation using bacterial or *A. oryzae nia*D sequences as probes. Chromosomal DNA was cleaved with *Pst*I which does not cut the recombinant clone, but does have a site in the polylinker (Fig. 3). The expected genomic *Pst*I fragment is 9.7 kb (Fig. 4). In summary, three types of hybridisation profile were observed, representative examples of which are shown in Fig. 6. First, nine transformants, such as TO27, lacked pUC sequences and had an *nia*D-hybridising *Pst*I band of approximately 9.7 kb which was indistinguishable from the recipient band (or the wild type, not shown). Such transformants were most likely a

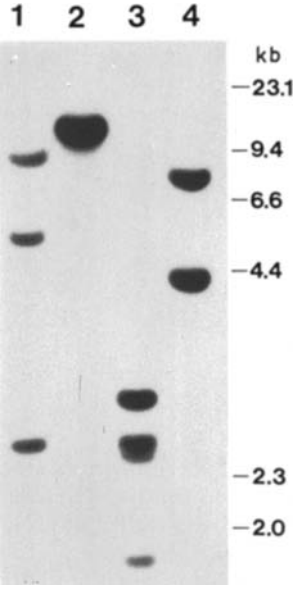

Fig. 4. Back hybridisation of pSTA14 to Southern blots containing digested genomic DNA. *Aspergillus oryzae* genomic DNA was digested with (1) *Eco*RI, (2) *Pst*I, (3) *Bam*HI and (4) *Bgl*II. Following electrophoresis and Southern blotting, hybridisation was carried out using *Pst*I-digested pSTA14 as probe at 65° C with washes to 0.2 × SSC. The positions of *Hin*dIII molecular size markers are indicated

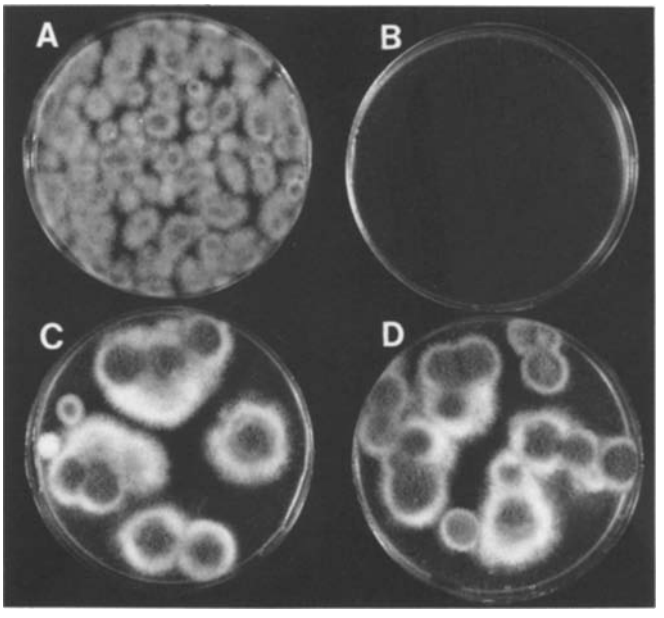

Fig. 5A–D. Appearance of control and transformed *nia*D14 cells on nitrate and ammonium. **A** Untransformed cells on the non-selective nitrogen source, ammonium; **B** untransformed cells on nitrate; **C** and **D** cells transformed with circular pSTA14 on nitrate

result of a gene conversion event. A second group exemplified by TO24 (five transformants) showed a single band of 12.0 kb hybridising to pUC while the resident *nia*D band was replaced by two other bands of 12.0 kb and 8.9 kb. It would seem that a single pSTA14 copy had integrated into the resident *nia*D site. Third, eight transformants, similar to TO26, were seen where the resident *nia*D band was

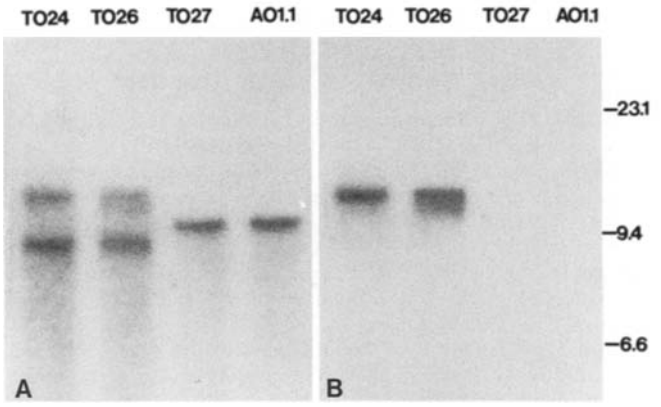

Fig. 6A and B. Analysis of transformants. Genomic DNA from three representative transformants and the recipient strain A01.1 was digested with *Pst*I and fragments separated on 0.8% agarose. After transfer to nylon, the blot was probed first with a ^{32}P-labelled, 5.5 kb *Hin*dIII fragment of pSTA14 (**A**). Following boiling twice for 10 min in distilled water containing 1% SDS, the blot was probed again with pUC18 (**B**) Hybridisation was carried out at 65° C with washes down to 0.2 × SSC. The positions of *λHin*dIII molecular size markers are indicated

Table 3. Co-transformation frequencies in nitrate-utilising transformants

System[a]	Co-transformation frequency (%)
*pyr*G[b]	4
*uid*A[c,d]	9
tub-2[c,e]	6

[a] For selection procedure, see Materials and methods. Screening for uracil auxotrophs was carried out on minimal medium lacking a pyrimidine
[b] Strain A04.1 (*nia*D14 *pyr*G1) used
[c] Strain A01.1 (*nia*D14) used
[d] Screening for co-transformants with β-glucuronidase activity was carried out on minimal medium containing 10 mM nitrate plus 20 μg/ml X-glucuronide
[e] Screening for benomyl resistant transformants was carried out at 1 μg/ml, a concentration which inhibits untransformed strains

replaced by three bands, border fragments of 12.0 kb and 8.9 kb as well as the band representing the unit length of pSTA14 (11.0 kb), indicating that more than one copy of pSTA14 had integrated in tandem at the *nia*D locus. No examples were seen of integration into a non-homologous site.

Heterologous transformation

Uncut pSTA14, containing the *A. oryzae nia*D gene, was introduced into stable *nia*D mutants of *A. nidulans* (*nia*D10), *A. niger* (*nia*D5) and *P. chrysogenum* (*nia*D19). The presence of functional copies of *A. oryzae nia*D was shown by phenotypic rescue of *nia*D non-reverting mutants of these fungi after heterologous transformation. Nitrate-utilising colonies of *A. nidulans*, *A. niger* and *P. chrysogenum* appeared at frequencies of 10, 43 and 2 transformants per microgram DNA respectively. Southern blots with vector pUC as probe showed that such transformants contained integrated bacterial sequences.

Co-transformation

The suitability of introducing unselectable markers into *A. oryzae* was assessed using the *A. oryzae pyr*G gene for repair of uracil auxotrophy (de Ruiter-Jacobs et al. 1989), *uid*A for β-glucuronidase (Roberts et al. 1989) and *N. crassa tub*-2 for benomyl resistance (Orbach et al. 1986) (Table 3). Co-transformation of all three genes was obtained as judged by phenotypic expression. Surprisingly perhaps, co-transformation frequencies were found to be rather low in all cases compared with, for example, the *pyr*G system in *A. oryzae* where using *pyr*G as the selectable marker, co-transformation frequencies were around 25% (de Ruiter-Jacobs et al. 1989).

Discussion

We have described an efficient homologous transformation based on the *nia*D system for *Aspergillus oryzae*, a filamentous fungus of considerable biotechnological interest. The level of transformation can approach 800 transformants per microgram DNA. Such events are single or multiple integrations and occur at the resident site. That the preponderance of integration events are single or low copy number at the natural site makes subsequent analysis and interpretation easier. Unlike certain other fungal transformation systems (Mattern et al. 1987; Hahm and Batt 1988; Iimura et al. 1987; Ballance and Turner 1985; Hynes 1986; Johnstone 1985), transformation with *nia*D results in a clean background with the complete absence of abortive transformants. We have also shown that it is possible to introduce non-selectable genes in to *A. oryzae* by co-transformation with *nia*D.

The nitrate system may be of general use for developing transformation in filamentous fungi for the following reasons. Nitrate reductase mutants have been generated and are available in a number of filamentous fungi (Unkles 1989) other than the well-characterised *A. nidulans* (Arst 1984; Cove 1979; Arst and Scazzocchio 1989; Kinghorn 1989). If not already available in other fungi of interest, it is straightforward to obtain *nia*D mutants. Indeed we easily generated *nia*D mutants in *A. oryzae*, *A. niger* and *P. chrysogenum* – without recourse to mutagenesis. Moreover, the rapid screening of chlorate resistant strains on the nitrogen sources described here conveniently and rapidly assigns mutants to gene loci. Large numbers of *nia*D mutants can be isolated, one of which at least is likely to be a stable deletion mutant strain suitable for transformation. Furthermore, we detected cross-hybridisation between *A. nidulans*, and *A. niger*, *A. oryzae* and *P. chrysogenum nia*D genes. At present these putative *nia*D genes are being used as the basis of a homologous system in these organisms and for heterologous transformation of other filamentous fungi that utilise nitrate. Finally, as the *A. oryzae nia*D gene is functionally expressed in three other fungi, *A. nidulans*, *A. niger* and *P. chrysogenum*, it may be possible that the nitrate system could be used as a universal transformation selection system for nitrate-assimilating fungi.

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A study was made of the integration events in eight transformants obtained using *Hin*dIII digested pSTA14. Analysis of Southern blots showed the presence of a single 9.7 Kb *nia*D-hybridising *Pst*I band in all transformants and no pUC-hybridising sequences. This indicates that as expected, transformation with *Hin*dIII-cleaved pSTA14 results only in gene replacements and eliminates bacterial sequences.