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A novel selectable marker based on *Aspergillus niger* arginase expression

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ABSTRACT

Selectable markers are valuable tools in transforming asexual fungi like *Aspergillus niger*. An arginase (*agaA*) expression vector and a suitable arginase-disrupted host would define a novel nutritional marker/selection for transformation. The development of such a marker was successfully achieved in two steps. The single genomic copy of *A. niger* arginase gene was disrupted by homologous integration of the *bar* marker. The *agaA* disruptant was subsequently complemented by transforming it with *agaA* expression vectors. Both *citA* and *trpC* promoters were able to drive the expression of arginase cDNA. Such *agaA*$^+$ transformants displayed arginase expression pattern distinct from that of the parent strain. The results are also consistent with a single catabolic route for arginine in this fungus. A simple yet novel arginine-based selection for filamentous fungal transformation is thus described.

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1. Introduction

Aspergillus niger is an industrially useful fungus capable of secreting a range of enzymes of commercial value. There is an increasing interest in Aspergilli as expression systems for heterologous proteins and protoplast mediated transformation is commonly employed to transform Aspergilli [1,2]. Both dominant and nutritional markers are used to select transformants. Cost effective nutritional markers are preferable but often mandate a suitable recipient background. This in turn requires that a critical anabolic gene (for instance, *argB* and *pyrG*) be mutated or disrupted. The *Aspergillus nidulans argB* gene (encoding ornithine transcarbamylase) can complement the corresponding auxotrophic mutation in *A. niger*. Homologous complementation by an auxotrophic marker is preferred in filamentous fungi. Accordingly, an *argB* disruption was created and complemented with its own *argB* gene in *A. niger* [3]. Available selection markers are not always suitable and an addition to the repertoire of markers is desirable. While the *argB* mediated selection exploits a critical anabolic step in arginine biosynthesis (Fig. 1), a few selection markers involving catabolic genes (*prn* and *pkiA*) are also known [4].

Arginase (the *agaA* gene product) provides the predominant catabolic route for L-arginine utilization in most fungi [5,6] and is a good candidate to establish as a nutritional marker. The recipient strain should be an *agaA* mutant for such a selection to work. The *agaA* mutants are difficult to score directly on media with or without L-arginine and require laborious replica plating. The metabolism of arginine, ornithine and proline in fungi are closely linked [6]. The biosynthesis of arginine and ornithine proceed by the acetylated pathway whereas proline can be synthesized by two routes (Fig. 1). Proline is formed from glutamate and mutations in this pathway result in proline auxotrophy. Such mutants are able to use arginine as a source of proline. This metabolic feature was exploited to select *agaA* mutants in two filamentous fungi [5,7]. Targeted arginase gene disruption offers a better alternative and was achieved in two strains of *Saccharomyces cerevisiae* [8,9]. The present work illustrates the construction of an *agaA* disrupted strain of *A. niger*. We also show that the arginase negative strain can be complemented with two different *agaA* expression vectors. A novel tool with arginase as a fungal transformation marker was thus defined.

2. Materials and methods

2.1. Strains and media

A. niger strain NCIM 565 (National Collection of Industrial Microorganisms, NCL-Pune, India) was used in this study. The BAR5 strain is a *bar*$^+$ transformant derived from *A. niger* NCIM 565 described previously [10]. Various fungal stock cultures and strains were maintained as slants on potato dextrose agar or YDA (yeast nitrogen base without amino acids and ammonium sulfate, dextrose and ammonium nitrate) agar supplemented with phosphinothricin (PPT; 1.0 mg/ml) (for *bar* transformants) or minimal medium (NM) agar supplemented with 2 mM arginine (for *agaA* transformants). PPT is a glutamine synthetase inhibitor and *bar* transformants are resistant to it. pCB1265 is a plasmid carrying *PtrpC-bar* [11]. Growth of *A. niger* and selection of *bar* transformants was done as described previously [10]. When required, NM was modified by replacing ammonium nitrate with appropriate nitrogen source.

2.2. Constructions to disrupt and express arginase

Cloning and propagation of plasmids were done in *Escherichia coli* XL1-Blue (Stratagene, CA, USA) and DH5α (Life Technologies, New Delhi, India) strains according to standard protocols [12]. The *E. coli* strain XL1 Blue was used in all the transformation experiments. Competent *E. coli* cells were prepared by PEG method

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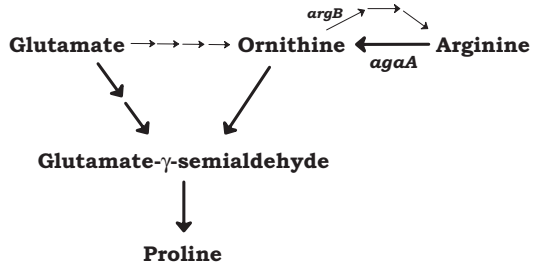


Fig. 1. Closely connected metabolism of arginine, ornithine and proline in filamentous fungi. Proline is an obligate intermediate in L-arginine catabolism and therefore arginine can serve as a source of proline in proline biosynthesis mutants. Bold arrows trace the two routes of proline synthesis. The schematic also depicts the steps of arginine biosynthesis (thin arrows).

Table 1
List of primers used in this work.

Primers	Sequence (5′–3′)	Sequence amplified
ArgMAF1	ctacatcggtgccgatctcgtggc	*agaA* gene
ArgMAR1	acaaccccatacagctggagcgat	*agaA* gene
BarPsF1	aactgcagatgagcccagaacgacgc	*bar* probe
BarXbR1	gctctagaaatctaaatctcggtgacgg	*bar* probe
CitNdR3	ggtggaagccatatggaatgtgaac	*PcitA*
DHCitF3	cataagcttggaacaccgtgcggc	*PcitA-aga* construction
FSDM	gcgaattccatatgacttctccttcgacaat	*aga* and *agac* probes
JPPstI	gccaagcgcgtcaagaacgc	*agac* probe and *PcitA-aga* construction
JPXhoIR	gctcgagcagaccggcttgaaacg	*aga* probe
TrpARF1	ccccacttgtaagcagtagcg	*PtrpC-aga* probe
TrpARR1	atggtaccgatagcgatggagt	*PtrpC-aga* probe
TrpCENR1	cggaattcatatgtacttcctaatcgaag	*PtrpC*
T3	aattaaccctcactaaaggg	*PcitA* probe, *PtrpC-bar* construction, *PcitA* and *PtrpC*

and stored in glycerol/PEG at −80 °C until further use. All the primers used for PCR in this study are listed in Table 1. These primers were from either Sigma–Aldrich (St. Louis, USA) or from Operon Biotechnologies Gmbh (Hilden, Germany).

The *agaA* gene was PCR-amplified from *A. niger* NCIM 565 genomic DNA using primers ArgMAF1 and ArgMAR1 (Table 1). The amplicon was cloned into a TA cloning vector (pTZ57R) to obtain pTZ57R-AF. The *PtrpC-bar* DNA for *bar* expression was PCR amplified from pCB1265 (using primers BarXbR1 and T3), processed and inserted within the *agaA* ORF in pTZ57R-AF (between *Eco*RV and *Xho*I) to obtain arginase disruption cassette pTZ57R-AF-Bar (Fig. 2A). While the *agaA* flanks (about 1.1 kb on both sides) in pTZ57R-AF-Bar were to facilitate homologous recombination, the selection of transformants is through *bar* selection. A functional *bar* gene confers PPT-resistance by acetylating and inactivating the herbicide.

A functional *citA* promoter (0.5 kb *PΔXcitA* DNA; [13]), named henceforth as *PcitA*, was PCR amplified (using primers T3 and CitNdR3, Table 1) and cloned in frame with *agaA* CDS [14] to obtain pΔXCA (Fig. 2B). Similar PCR strategy (with primers T3 and TrpCENR1) was used to clone *PtrpC* in frame with *agaA* CDS to obtain pBSPtrpCaga (Fig. 2C).

In all PCR reactions – 3.5 mM $MgCl_2$, 250 μM dNTP's, 0.5 μM primers and 5 units of *Taq* polymerase (New England BioLabs, Ipswich, USA) or *Pfu* Polymerase (MBI Fermentas, St. Leon-Rot, Germany) were present in 100 μl of the final PCR mixture. Genomic PCR being very sensitive to template DNA concentrations, 200 ng of *A. niger* genomic DNA was used for *agaA* amplifications. The plasmid DNA concentration of 20–30 ng was used for PCR amplification (30 cycles). Restriction enzymes used during the construction of various expression vectors were from New England BioLabs or MBI Fermentas. DNA fragments were extracted from agarose gel pieces using QIAquick columns (from QIAGEN Gmbh, Hilden, Germany) and Nucleospin extract II columns (from Macherey-Nagel, Düren, Germany) according to procedure recommended by the supplier. T4 DNA ligase was from MBI Fermentas. Ligation mixtures contained 500 ng DNA in 50 μl reaction volume and were performed at 22 °C for 4 h.

2.3. Aspergillus transformation and agaA selection

A. niger mycelia grown in liquid NM (10^8 spores per 100 ml medium in shake flasks for 16 h, at 30 °C) were harvested on cheese cloth (3–4 h after mycelial branching begins). Protoplast preparation, transformation, selection of transformants and their mitotic stability check were performed as before [10].

The selection of *agaA* knockouts was done in two stages. First the *bar* transformants were selected on PPT plates. All these were subsequently replica plated on media with L-arginine and L-ornithine (as sole nitrogen sources). The *bar* transformants that grew on L-ornithine plates but not on arginine plates were selected for further analysis. Mitotically stable, putative *agaA* disruptants (*agaA::bar*) were characterized by growth on NM agar containing L-arginine (5 mM), L-ornithine (10 mM) or urea (10 mM). One such *agaA* disrupted strain (strain D-42) served as a recipient and was complemented with *agaA* expression vector (see below).

The *agaA*$^+$ transformants (obtained from D-42 strain), were selected directly on NM agar supplemented with sucrose (1.2 M). More importantly, arginine (2 mM) replaced ammonium nitrate as the sole nitrogen source in these plates. The *agaA*$^+$ transformants were propagated and maintained on NM with arginine as the sole nitrogen source (NM + Arg medium). Homologous integration at the *agaA* locus was inferred from inability to grow on PPT plates (*bar*$^-$ phenotype).

The Southern analysis was performed as mentioned previously [10]. Suitable *bar* (571 bp fragment amplified from pCB1265 with primers BarPsF1 and BarXbR1), *aga* (253 bp arginase ORF fragment amplified from pTZ57R-AF with primers FSDM and JPXhoIR), *PcitA* (508 bp *Nde*I-*Xho*I fragment isolated from the T3-CitNdR3 amplicon of pΔXCA), *PtrpC-aga* (598 bp, amplified from pBSPtrpCaga with primers TrpARF1 and TrpARR1) and *agac* (523 bp, arginase cDNA amplified from pBSPtrpCaga with primers FSDM and JPPstI) amplicons were digoxigenin-labeled according to manufacturer's (Roche Applied Science) instructions and used as probes (at 10 ng/ml).

2.4. Arginase assay

A. niger mycelia were extracted with arginase extraction buffer (200 mM imidazole HCl, 2 mM 2-mercaptoethanol, 1 mM PMSF, 12 mM $MnSO_4$, pH 7.5) [15]. Arginase was assayed by estimating the urea formed [14]. One unit of arginase activity is defined as 1 μmol of urea formed in 1 min in the standard assay. Specific activity is defined as units per mg of protein. Protein was estimated according to Bradford [16].

The native polyacrylamide gel electrophoresis (PAGE [17]) was performed with 10% resolving gel and a 5% stacking gel; proteins were stained with Coomassie Blue R-250. Stained PAGE gels were analyzed with Geliance 1000 2D Imaging system (Perkin Elmer, CA, USA) with GeneSnap software version 7.04. The *A. niger* arginase protein, heterologously expressed in and purified from *E. coli* [14], served as a positive control.

3. Results and discussion

3.1. Disruption of A. niger arginase

The arginase gene from *A. niger* NCIM 565 (GeneBank Acc. No. AF242315) was sequenced and characterized [14]. While mutations and/or disruption of the arginase gene are valuable in understanding fungal physiology, these are not available for *A. niger*. Classical mutagenesis and screening by replica plating was employed to obtain arginase mutants of *A. nidulans* and *Neurospora crassa* [5,7,18]. Targeted gene disruption through homologous recombination provides an alternative way to obtain near isogenic knockout strains. Among fungi, targeted arginase gene disruption is reported for yeast [8,9]. Low frequency of homologous recombination and the need for long flanking regions of homology limit efficient gene targeting in filamentous fungi [1,19]. Our initial attempts at disruption using a short arginase cDNA fragment (a 0.7 kb *Eco*RI–*Sal*I fragment inserted in the multiple cloning site of pCB1265) were unsuccessful (none out of 200 *bar* transformants isolated). Availability of the complete sequence of the *A. niger* genome (http://genome.jgi-psf.org/Aspni1/Aspni1.home.html) facilitated the PCR cloning of *agaA* gene with its flanking sequences (3.4 kb DNA in pTZ57R-AF). The 1.1 kb 5′ upstream *agaA* gene sequence from *A. niger* NCIM 565 contained the highly conserved ARCA binding sequence (ctctcctgggcctgtacttaggggaacaaccgttcc-ctaaaagcaggtcttagcgat), as reported for the *agaA* and *otaA* (L-ornithine δ-transaminase) promoter regions of *A. nidulans* [20,21].

A disruption vector (pTZ57R-AF-Bar) for homologous recombination at the *agaA* locus was developed from pTZ57R-AF (Fig. 2 and Section 2). *A. niger* NCIM 565 was transformed with *Sma*I-linearized pTZ57R-AF-Bar. Out of 234 *bar* transformants screened, three (designated as D-42, D-52 and E-95) did not grow on NM + Arg plates. However, they showed normal growth on L-ornithine and urea. These three potential arginase disruptants were further characterized. Apart from its poor growth on NM, strain E-95 also showed

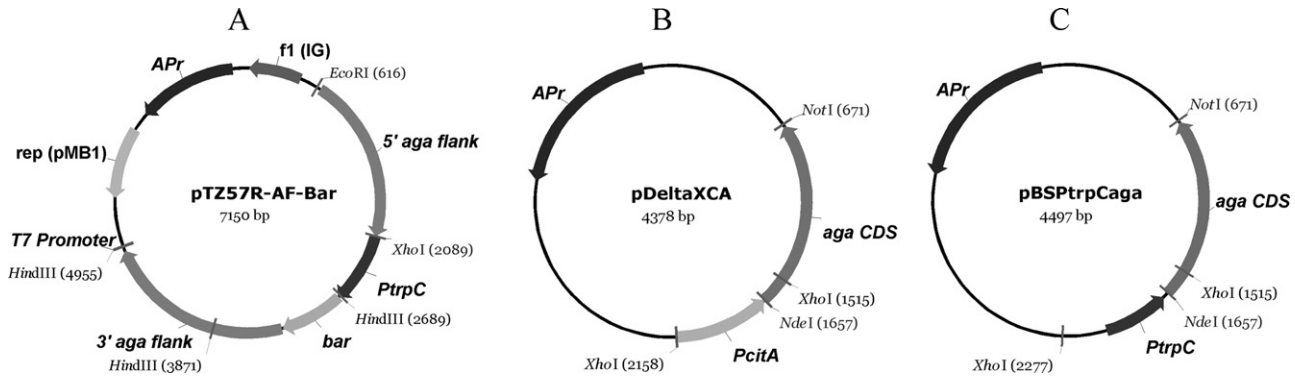


Fig. 2. Arginase disruption and expression vectors. The *bar* marker was inserted within arginase gene (A) for disruption. The two promoters namely *PcitA* (B) and *PtrpC* (C), were used to drive arginase expression.

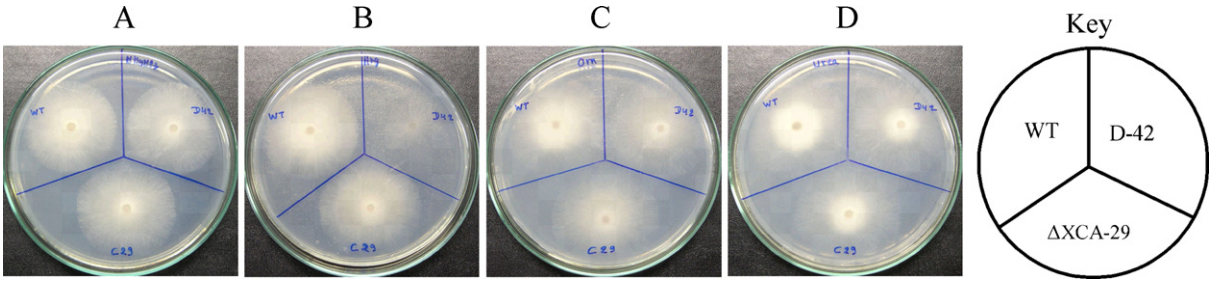


Fig. 3. Growth of *A. niger* strains on different nitrogen sources. Spores of putative arginase knockout (strain D-42), parent (strain NCIM 565) and an *agaA* transformant (strain ΔXCA-29) were spot inoculated on NM medium (A), or NM without ammonium nitrate but supplemented with 5 mM L-arginine (B), 10 mM L-ornithine (C) or 10 mM urea (D) as sole nitrogen source. Growth was recorded after 60 h.

scant conidiation and poor hyphal development. It grew normally on L-ornithine-supplemented media suggesting a possible anomaly in L-ornithine metabolism. Further characterization of E-95 is in order. Comparative growth of D-42 strain and *A. niger* NCIM 565 (parent strain) on different nitrogen sources is shown in Fig. 3. An *agaA* disrupted strain is not expected to grow on NM with arginine as sole nitrogen source but should grow normally on NM or NM modified with ornithine or urea (the two products of arginase reaction). The growth pattern on different nitrogen sources was consistent with *agaA* disruption in D-42 strain.

Arginase enzyme activity in the three transformants D-42, D-52 and E-95 was evaluated. Comparable growth of the three strains was ensured on NM with L-proline (50 mM) as the sole nitrogen source (also, L-proline is a non-repressible nitrogen source). No arginase activity was detected in the mycelial extracts of these three transformants, when grown on media containing proline or proline + arginine. This result was independent of whether the medium contained 15 mM L-arginine or not, suggesting that they may not be regulatory mutants. As expected, both *A. niger* NCIM 565 (parent strain) and BAR5 (the *bar*$^+$ control strain) showed normal and induced (fivefold) arginase activity. The arginase activity data for the BAR5 strain (*A. niger* NCIM 565 with a single copy pCB1256 integration [10]) suggested that expression of arginase was independent of the presence or absence of *bar* cassette in the genome. The absence of arginase activity in D-42 crude extracts was also corroborated by the native PAGE data; the protein band corresponding to arginase was absent (see below).

Integration events in the three *agaA* disruptants were analyzed through Southern analysis. Restriction enzyme digests (*Bam*HI and *Hin*dIII) of genomic DNA from strains D-42, D-52 and E-95 were probed with two different probes (*aga* and *bar*; Section 2). As expected, the *aga* probe picked up a single DNA fragment (*Hin*dIII digestion) from the three *agaA* disruptants as well as from the parent strain. The *Bam*HI digest of the parent strain showed two bands (1.1 kb and 1.8 kb; for a schematic see Fig. S1). However, in the three *agaA* disruptants the larger band was of 2.6 kb (Fig. 4). The Southern data is consistent with the presence of an additional *bar* cassette in these strains. The 2.6 kb fragment also hybridized with the *bar* probe (not shown).

A combination of growth studies, arginase protein and activity and Southern analyses point to successful disruption of the arginase gene in *A. niger* by homologous recombination. Despite large homologous flanking sequences employed, the frequency of *A. niger agaA* disruptants obtained was very low (three out of 234 *bar* transformants). Targeted disruption was shown to be best achieved by employing a Δ*kusA* strain (defective in Non-Homologous End Joining pathway for DNA repair) in which homologous recombination predominates [22]. *A. niger* NCIM 565 employed in this study does not have this Δ*kusA* genetic background.

While E-95 grew and sporulated poorly on NM, the integration pattern in D-52 strain was different (larger than the expected 2.6 kb fragment release). On these grounds, of the three *agaA* knockouts (D-42, D-52 and E-95), D-42 was the best choice. *A. niger* D-42 strain is the first example of targeted arginase disruption in a filamentous fungus and all our further studies were confined to this strain.

3.2. Arginase as a novel selection marker for transformation

A review of relevant fungal metabolism suggests that arginase is the sole route for L-arginine catabolism [5,6]. Another possible route for L-arginine metabolism is through its decarboxylation to agmatine. Agmatinase, a member of this L-arginine catabolic pathway, is not reported from fungi. Agmatinase activity in *A. niger* extracts could not be demonstrated (unpublished observations). Under the growth conditions tested, *agaA* disrupted strain of *A. niger* was able to grow on ornithine

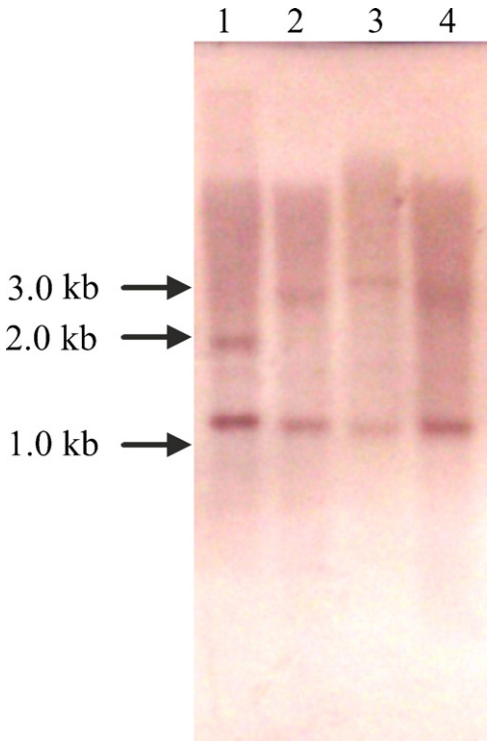
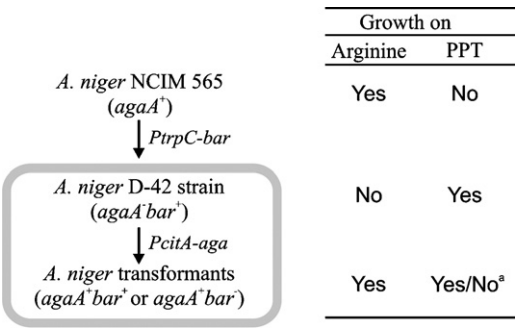


Fig. 4. Southern analysis of *A. niger agaA* disruptants. The *Bam*HI digested genomic DNA from *A. niger* NCIM 565 (Lane 1), strain D-42 (Lane 2), strain D-52 (Lane 3) and strain E-95 (Lane 4) were hybridized to *aga* probe.



	Growth on	
	Arginine	PPT
A. niger NCIM 565 (*agaA*$^+$)	Yes	No
A. niger D-42 strain (*agaA*$^-$*bar*$^+$)	No	Yes
A. niger transformants (*agaA*$^+$*bar*$^+$ or *agaA*$^+$*bar*$^-$)	Yes	Yes/No[a]

Fig. 5. Strategy for *agaA*-based selection of transformants. The *agaA* disrupted *A. niger* (D-42 strain) does not grow on arginine plates. When D-42 strain is transformed with *PcitA-aga* gene construction, transformants gain the ability to grow on arginine plates. In principle, these transformants could be of two types[a] – those growing on PPT plates (*bar*$^+$) and those not growing on PPT plates (*bar*$^-$) – depending on whether homologous recombination has occurred or not.

or urea as the sole nitrogen source, but not on arginine (Fig. 3). Lastly, our in silico analysis predicted a single copy of arginase gene in the *A. niger* genome (http://genome.jgi-psf.org/Aspni1/Aspni1.home.html). That arginase defines the only route for L-arginine catabolism (Fig. 1), created an excellent opportunity to establish a nutritional selection system. A suitable arginase expression vector (along with D-42, an *agaA* disrupted strain) was used to define a new marker for selection. The *agaA*-based selection to score *A. niger* transformants is shown in Fig. 5. This strategy could be demonstrated as follows. The D-42 strain was transformed with a *PcitA-aga* carrying plasmid (*Sca*I linearized pΔXCA; see Section 2) and the transformants were selected on NM with L-arginine (2 mM) as sole nitrogen source. The recipient D-42 strain did not grow on this medium while the transformants could be scored successfully (Fig. 6). A total of 49 stable transformants were isolated and single-spored on arginine-selection plates. In principle, these transformants fall into two groups – *agaA*$^+$*bar*$^+$ and *agaA*$^+$*bar*$^-$ (Fig. 5). Therefore, all the *agaA*$^+$ transformants were tested for their ability to grow on PPT plates as well. Three of them (strains ΔXCA-1, ΔXCA-4 and ΔXCA-7) did not grow on PPT plates, possibly examples of homologous integration. The *agaA*$^+$ transformants showed a range of relative arginase specific activities (1.5–22.0 U/mg as compared to 4.0 U/mg in *A. niger* NCIM 565). The enhanced arginase specific activity in some of these transformants could be due to multi-copy integrations (as seen from Southern analysis of *Hin*dIII genomic DNA digests probed with *PcitA* probe).

Three representative *agaA*$^+$ transformants were selected for further study. Two of them were *agaA*$^+$*bar*$^+$ (ΔXCA-24, arginase expression comparable to *A. niger* NCIM 565 and ΔXCA-29, high arginase expression) and one was *agaA*$^+$*bar*$^-$ (ΔXCA-7). The integration of *PcitA-aga* DNA in these transformants was confirmed through genomic PCR (with primers DHCitF3 and JPPstI; data not shown) and Southern analysis using the *PcitA* probe, specific to the promoter region of the expression vector. As expected, the probe picked up the genomic copy of *citA* and the 643 bp DNA fragment (released by *Xho*I) of *PcitA-aga* integrated in these transformants (Fig. S2).

Nitrogen metabolite control is known to operate in Aspergilli. While ammonium is the preferred nitrogen source, arginine is an inducer of arginase in *A. nidulans* [18,23]. Induction (fivefold over the basal level) of *A. niger* arginase activity by L-arginine (Table 2) is consistent with the notion that *PagaA* is an inducible promoter. On the other hand, arginase expression under *PcitA* control is not expected to be induced by L-arginine. The arginine inducible feature of the three *agaA*$^+$ transformants was tested by growth on NM with and without L-arginine as sole nitrogen source. All three strains showed growth comparable to that of *A. niger* NCIM 565. Although

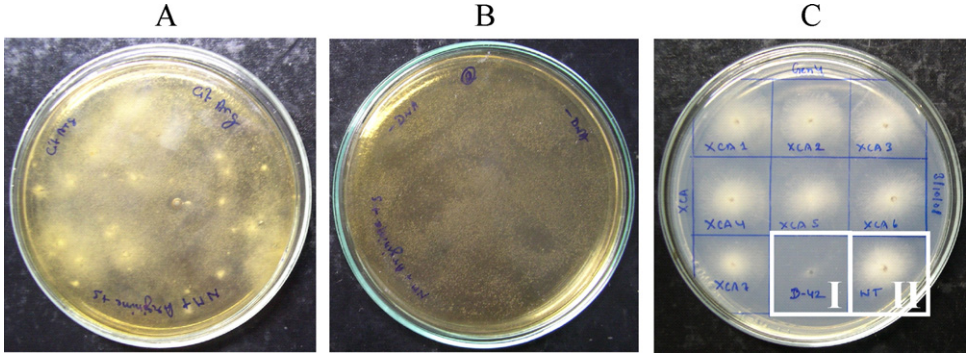


Fig. 6. Selection of *agaA* transformants on arginine plates. The D-42 strain was transformed with linearized *PcitA-aga* carrying plasmid and the transformants selected on arginine plate (A). Plate with no-DNA control is shown in (B). Selected transformants were passaged on arginine plates for seven generations and subsequently spot inoculated on the same medium (C); their growth was compared with strain D-42 (I) and *A. niger* NCIM 565 (II).

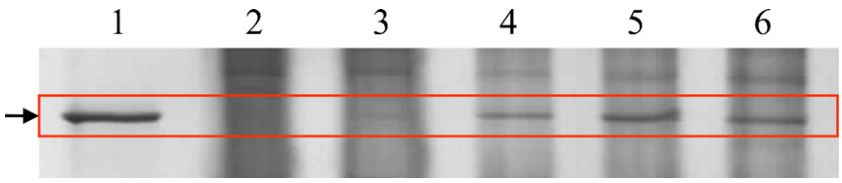


Fig. 7. Monitoring arginase protein in different *agaA* transformants. Total soluble protein extracted from *A. niger* mycelia grown on NM (D-42 strain, Lane 2; *A. niger* NCIM 565, Lane 3; ΔXCA-29 strain, Lane 4) and NM + Arg (*A. niger* NCIM 565, Lane 5; ΔXCA-29 strain, Lane 6) media were resolved on native PAGE. Equal amount of protein (40 μg) was loaded in Lanes 2–6. Lane 1 contains pure *A. niger* arginase protein and the protein bands were visualized by Coomassie Blue staining.

Table 2
Relative arginase activities of different *agaA*$^+$ transformants.

A. niger strain	Phenotype	Specific activity[a] (U/mg)		Fold induction
		NM	NM + Arg	
NCIM 565	*agaA*$^+$	4.3	22.9	5.3
D-42 (*agaA::bar*)	*agaA*$^-$*bar*$^+$	ND[b]	ND	–
ΔXCA-7	*agaA*$^+$*bar*$^-$	1.6	2.5	1.6
ΔXCA-24	*agaA*$^+$*bar*$^+$	5.0	5.6	1.1
ΔXCA-29	*agaA*$^+$*bar*$^+$	22.1	22.6	1.0
TrpArgC8[c]	*agaA*$^+$*bar*$^+$	3.7	4.8	1.3

[a] Specific activity data is best of three independent experiments; relative arginase activity from mycelia grown on NM and NM + Arg (NM with arginine as sole nitrogen source) was monitored.
[b] ND, No activity detected.
[c] Arginase activity in TrpArgC8 strain (transformant obtained with *PtrpC-aga* construction) was comparable when grown on NM, NM + Arg (arginine, 14 mM) and NM + Trp (tryptophan, 5 mM; not shown).

different basal levels were observed, relative arginase specific activity was unaffected even when L-arginine was the sole nitrogen source (Table 2). The constitutive nature of arginase expression was also corroborated by the native PAGE data (Fig. 7). The *PcitA* driven arginase expression was thus not under arginine control, an observation consistent with the expectation that *PcitA* displays strong and constitutive expression [13].

The *agaA* nutritional selection in *A. niger* was also demonstrated using a different promoter to drive arginase expression (*PtrpC-aga*). For this, *A. niger* D-42 strain was transformed with linearized (*Sca*I) pBSPtrpCaga and the transformants were selected on arginine plates as before (Fig. 6). Of the 8 stable *agaA* transformants isolated (purified by repeated single-sporing on arginine plates), none were homologous recombinants (i.e. *bar*$^-$ phenotype). Two *PtrpC-aga* transformants (TrpArgC8 and TrpArgC9), when further characterized, showed healthy growth and good sporulation. The genomic integration of *PtrpC-aga* DNA in them was confirmed through diagnostic genomic PCR and Southern analysis (not shown). *PtrpC* driven arginase expression (in TrpArgC8 transformant) was independent of the presence of arginine or tryptophan in the growth medium (Table 2). In the arginase-less background of D-42 strain, and as exemplified with two different promoters (*PtrpC* and *PcitA*), arginase can thus serve a fungal transformation marker.

4. Conclusions

We successfully disrupted the *agaA* gene in *A. niger* and used this genetic background to demonstrate a novel nutritional selection through arginase complementation. The only other marker based on fungal arginine metabolism is *argB* – a typical auxotrophic marker [3]. The corresponding recipient strain for *argB* should be a strict L-arginine auxotroph that cannot grow on minimal medium. In contrast, *agaA* marker is unique where the recipient is a conditional auxotroph. Being a catabolic enzyme, arginase is indispensable only when L-arginine is the sole nitrogen source. The *agaA* marker described here offers a simple yet tight selection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2012.04.001.

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