A novel expression system for intracellular production and purification of recombinant affinity-tagged proteins in *Aspergillus niger*.

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Abstract

A set of different integrative expression vectors for the intracellular production of recombinant proteins with or without affinity tag in *Aspergillus niger* was developed. Target genes can be expressed under the control of the high-efficient, constitutive *pkiA* promoter or the novel sucrose-inducible promoter of the β-fructofuranosidase (*sucA*) gene of *A. niger* in the presence or absence of alternative carbon sources. All expression plasmids contain an identical multiple cloning sequence that allows parallel construction of N- or C-terminally His_6- and StrepII-tagged versions of the target proteins. Production of two heterologous model proteins, the green fluorescence protein (GFP) and the *Thermobifida fusca* hydrolase (TFH), proved the functionality of the vector system. Efficient production and easy detection of the target proteins, as well as their fast purification by a one-step affinity chromatography, using the His_6- or StrepII-tag sequence, was demonstrated.

Introduction

Aspergillus niger has many applications in various biotechnical and industrial processes, such as the production of organic acids (e.g. citric acid, gluconic acid). In addition, a large variety of complex enzymes and recombinant proteins have been successfully produced by *A. niger*, proficient to perform eukaryotic posttranslational modifications, i.e. glycosylation and proteolytic processing. Among them are the phytase (Shah et al. 2008), the xylanase (Bakri et al. 2008), the glycosylated fructofuranosidase SucA (Zuccaro et al. 2008), the laccase from *Trametes versicolor* (Bohlin et al. 2006), the tissue plasminogen activator (t-PA) (Wiebe et al. 2001), humanized antibodies (Ward et al. 2004), human lymphotoxin α (Krasevec et al. 2000), and the glycosylated alpha1-proteinase (Chill et al. 2009; Karnaukhova et al. 2007) to name a few and to emphasize the enormous potential of this organism. Products of *A. niger* are generally regarded as safe (GRAS status) and are used as food additives, bread improver and therapeutic substances.

Although *A. niger* has long been exploited commercially for metabolite production, high level synthesis of most heterologous proteins was very difficult. However, in recent years, protease-deficient strains, transformation strategies and crucial genetic tools have been developed to enhance recombinant expression of specific proteins by *A. niger*. Some integrative and autonomously replicating vector systems for *A. niger* with selective markers have been established to clone and express heterologous genes (Punt and van den Hondel 1992; Storms et al. 2005; Verdoes et al. 1994). An expression system, that has been exploited for the production of most recombinant proteins in *A. niger* utilizes the maltose-inducible promoter of the *glaA* gene, encoding the highly expressed and efficiently secreted enzyme glycoamylase (Fowler et al. 1990).

Nevertheless, the development of strains suitable for high-level protein production is still limited by the low number of different inducible expression vectors in comparison to other fungal model systems, such as *Saccharomyces cerevisiae*, and *Pichia pastoris*. The most frequently used P_{glaA} promoter is slightly expressed even under non-inducing conditions (Davies 1994) and might thus be not suitable for the production of recombinant proteins that are deleterious or toxic for *A. niger*. Furthermore, no plasmid system is yet available for *A. niger*, which can be used for overexpression of proteins tagged with small ligand-binding peptides to allow easy detection and efficient purification of the recombinant products. Polyhistidine (His-tag) and streptavidin (StrepII-tag) binding peptides are commonly used, as they generally have no influence on protein function and offer high yields of purified proteins at moderate costs (Lichty et al. 2005). In this work, we constructed a set of integrative plasmid vectors, that can be used for constitutive and inducible expression of tagged and untagged recombinant proteins in *A. niger*. The new expression vectors were successfully tested with two different heterologous model proteins, the green fluorescence protein variant S65TGFP with an increased fluorescence and a faster auto-oxidative fluorophore formation (Cubitt et al. 1995; Ganzlin and Rinas 2008; Heim et al. 1995), and the extracellular hydrolase from *Thermobifida fusca* (TFH) (Dresler et al. 2006).

Materials and Methods

Strains, media and culture conditions

Amplification of plasmids was routinely performed in *Escherichia coli* strains Top10, DH10B and ER2925, listed in Table 1. The protease-deficient (*prt*) *A. niger* strain AB1.13 *prt-13* (Mattern et al. 1992), used to test the expression system, is an UV

irradiated derivative of AB4.1 (van Hartingsveldt et al. 1987), a *csp*A1 *pyr*G1 derivative of strain ATTC 9029 (Bos et al. 1988).

Mycelia were routinely grown from conidia in a modified minimal medium (Pontecorvo et al. 1953), including 11 mM KH_2PO_4, 100 mM of the appropriate carbon source and 1 ml l^{-1} trace element solution (Vishniac and Santer 1957). 10 mM uridine was added for the cultivation of the *pyrG* deficient *A. niger* strain AB1.13. Routinely, 30 ml of culture medium were inoculated with $3 \cdot 10^7$ conidia and grown at 37°C under aeration (140 rpm).

DNA manipulation for vector constructions

Standard DNA manipulations, plasmid DNA isolation and transformation in *E. coli* were performed as described (Sambrook and Russell 2001).

Plasmids and primers used for DNA sequencing and vector construction in this study are listed in Table 1 & Table S1. The basic integrative vector ANIp8 for intracellular production of recombinant proteins in *A. niger* was derived from vector ANEp8 (Storms et al. 2005). First, the AMA1 sequence supporting autonomous replication was removed from ANEp8 by *Not*I digest and religation. Oligonucleotides MCS-*Nco*I-for and MCS-*Nco*I-rev including the multiple cloning site MCS1 were annealed and the generated DNA fragment was inserted into the *Nhe*I/*Fse*I sites of ANIp8 to generate pARAn8 (P_{pkiA}-MCS1). The *Aat*II-*Nhe*I fragment harbouring the *pki*A promoter was exchanged against a PCR generated fragment amplified with primers P_{sucA}-*Aat*II and P_{sucA}-*Nhe*I for the construction of pARAn1 (P_{sucA}-MCS1), including the inducible *A. niger sucA* promoter.

Based on the vector sequence of ANIp8 two oligonucleotides new-MCS-for and new-MCS-rev with eight unique restriction sites (MCS2) were designed, annealed and cloned into the *Nhe*I/*Fse*I site of pARAn8 and pARAn1 to generate vectors

pARAn9 (P_{pkiA}-MCS2) and pARAn11 (P_{sucA}-MCS2). The *gfp* expression vector was constructed by insertion of a PCR generated fragment, amplified from pAN56-2*gfp* (Gordon et al. 2000) with primers PCR-*gfp*-*Afl*II-for and PCR-*gfp*-*Cla*I-rev, into the *Afl*II/*Cla*I sites of pARAn1 and pARAn8 to generate pARAn101 (P_{sucA}-*gfp*) and pARAn801 (P_{pkiA}-*gfp*). Introduced sequences of all plasmids were verified by sequencing (GATC, Germany) using the sequencing primers seq-P_{sucA}-for or seq-P_{pkiA}-F and seq-ANIp8-MCS-rev listed in Table S1.

Construction of His_6-tagged expression vectors

The His_6-tag sequence was adapted to the codon usage of *A. niger* by using the software JCat (Grote et al. 2005). Fragments, encoding a C-terminally His_6-tagged GFP variant, were produced by PCR with primers *gfp*-*Bss*HII-*Eco*RI-for and *gfp*-*Eco*RI-H-*Fse*I-rev and inserted into the *Bss*HII/*Fse*I sites of pARAn9 and pARAn11, generating pARAn18 (P_{pkiA}-*gfp*-His_6) and pARAn17 (P_{sucA}-*gfp*-His_6). C-terminally His_6-tagged constructs of the *tfh* gene, generated from vector pYYBm2 (Yang et al. 2007) with primers *tfh*-*Bss*HII-*Eco*RI-for and *tfh*-*Eco*RI-His-*Fse*I-rev, were introduced into the *Bss*HII/*Fse*I sites of pARAn9 and pARAn11, resulting in pARAn15 (P_{pkiA}-*tfh*-His_6) and pARAn16 (P_{sucA}-*tfh*-His_6). For the construction of the basal C-terminal His-tag vectors, the *Eco*RI fragment, encoding the TFH, was removed from pARAn15 and pARAn16 and religated to generate pARAn19 (P_{pkiA}-MCS2-His_6) and pARAn20 (P_{sucA}-MCS2-His_6).

The N-terminal His_6-tag *tfh* constructs were amplified with primers *tfh*-*Cla*I-*Age*I-His-for and *tfh*-*Bsr*GI-*Age*I-rev and inserted into the *Cla*I/*Bsr*GI sites of pARAn9 and pARAn11 generating pARAn27 (P_{pkiA}-His_6-*tfh*) and pARAn29 (P_{sucA}-His_6-*tfh*). For the construction of the basal N-terminal His-tag vectors, the *Age*I fragment encoding the

tfh gene was deleted from pARAn27 and pARAn29 and religated to generate pARAn31 (P_{pkiA}-His_6-MCS2) and pARAn32 (P_{sucA}-His_6-MCS2).

Construction of StrepII-tagged expression vectors

For the production of proteins harbouring a StrepII-tag with the amino acid sequence WSHPQFEK, we used oligonucleotides, that encode C- (C-Strep-for, C-Strep-rev) or N-terminal tags (N-Strep-for, N-Strep-rev). The StrepII-tag sequences were also optimized according to the codon usage of *A. niger* (Grote et al. 2005). The C-terminal StrepII oligonucleotides were annealed and inserted into the *Eco*RI/*Fse*I sites of pARAn11, which resulted in the generation of pARAn33 (P_{sucA}-MCS2-StrepII). Vector pARAn36 (P_{pkiA}-MCS2-StrepII) was constructed by substitution of the *Aat*II/*Nhe*I fragment in pARAn33, containing P_{sucA} against the *Aat*II/*Nhe*I fragment, including P_{pkiA} of pARAn9.

Similarly, N-terminal StrepII oligonucleotides were annealed and inserted into the *Cla*I/*Age*I sites of pARAn11, resulting in pARAn34 (P_{sucA}-StrepII-MCS2). For the construction of pARAn37 (P_{pkiA}-StrepII-MCS2), the *Aat*II/*Nhe*I fragment of pARAn34 was exchanged against the P_{pkiA} containing *Aat*II/*Nhe*I fragment of pARAn9. To test the expression of N- or C-terminally StrepII-tagged proteins, the *gfp* gene was amplified with primers *gfp*-*Cla*I-H-AgeI-for and *gfp*-FseI-rev and inserted into the *Age*I/*Fse*I sites of pARAn33 to generate pARAn47 (P_{sucA}-*gfp*-StrepII). In order to introduce the *gfp* gene into the *Age*I/*Eco*RI sites of pARAn36, it was amplified with the primers *gfp*-*Cla*I-H-*Age*I-for and *gfp*-*Eco*RI-H-*Fse*I-rev and the resulting plasmid was named pARAn60 (P_{pkiA}-*gfp*-StrepII). An *Eco*RI-fragment harbouring the *tfh* gene was isolated from pARAn16 and inserted into the *Eco*RI site of pARAn36 to generate pARAn63 (P_{pkiA}-*tfh*-StrepII).

An additional set of *gfp* expression vectors was constructed in which a stop codon was introduced downstream of the *Eco*RI site following the *gfp* coding sequence. To do so, the *gfp* gene was amplified by PCR using the primers *gfp-Bss*HII-*Eco*RI-for and *gfp-Eco*RI-Stop-*Fse*I-rev and cloned into the *Bss*HII/*Fse*I sites of pARAn9, pARAn11, pARAn31, pARAn32, pARAn34 and pARAn37, which resulted in the vectors pARAn49 (P_{pkiA}-*gfp*-stop), pARAn54 (P_{sucA}-*gfp*-stop), pARAn53 (P_{pkiA}-His_6-*gfp*-stop), pARAn55 (P_{sucA}-His_6-*gfp*-stop), pARAn56 (P_{sucA}-StrepII-*gfp*-stop) and pARAn50 (P_{pkiA}-StrepII-*gfp*-stop). Subsequently, the *gfp* coding sequence was removed by digestion of flanking *Eco*RI sites, and the following vectors were generated by religation: pARAn51 (P_{pkiA}-MCS2-stop), pARAn58 (P_{sucA}-MCS2-stop), pARAn57 (P_{pkiA}-His_6-MCS2-stop), pARAn59 (P_{sucA}-His_6-MCS2-stop), pARAn61 (P_{sucA}-StrepII-MCS2-stop) and pARAn52 (P_{pkiA}-StrepII-MCS2-stop). The complete nucleotide sequences of the basal expression vectors pARAn51 (P_{pkiA}-MCS-stop) and pARAn58 (P_{sucA}-MCS-stop) were submitted to the NCBI GenBank database (pARAn51: Acc. No. FJ868796, pARAn58: Acc. No. FJ868795).

Transformation of *A. niger*

Preparation of protoplasts and transformation of *A. niger* was performed as previously described (Punt and van den Hondel, 1992) with the exception that 20 mg/ml lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, Munich, Germany) and 2,5 mg/ml of Driselase from *Basidomycetes sp.* (Sigma-Aldrich, Munich, Germany) were used for protoplastation. Uracil auxotrophy was used for selection, and transformed protoplasts were plated onto minimal medium plates, including 1.2% (w/v) agar and 1.2 M sorbitol, using agar overlays of minimal medium with 0.6% (w/v) agar and 1.2 M sorbitol. Colonies were grown at 37°C for 3-4 days, and purified three times on minimal medium plates.

Analysis of recombinant protein production

To determine the intracellular amount of recombinant proteins, *A. niger* strain ARAn101 was grown under standard conditions for 70 h with 100 mM of the different carbon sources. For the time course of *gfp* expression, strain ARAn101 was grown for 48 h before induction with 25 mM sucrose. The biomass was separated from the medium by filtration (Whatman, Dassel, Germany, cellulose filters 2.5 µm). Approximately 200 mg wet weight (WW) were given to a Matrix E beat beater tube (MP Biomedicals, Eschwege, Germany) with 1 ml fluorescence buffer (200 mM NaCl, 50 mM Na_2HPO_4, 10 mM Tris-HCl pH 8) and treated three times for 45 sec with 6 m s^{-1} in the beat beater FastPrep®-24 (MP Biomedicals, Eschwege, Germany).

GFP was visualized by western blotting with a rabbit anti-GFP antibody (Acris, Herford, Germany) and a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Sigma). Strep-tagged proteins were detected by a mouse antibody conjugated with alkaline phosphatase directed against the StrepII-tag (Strep-Tactin®, IBA GmbH, Göttingen, Germany). His_6-tagged proteins were visualized with a monoclonal mouse antibody against the His_6-tag (Qiagen, Hilden, Germany) and a goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Sigma-Aldrich, Munich, Germany) was used as secondary antibody.

Detection of the green fluorescent protein (GFP) and the hydrolase from *Thermobifida fusca* (TFH)

GFP expressed in *A. niger* was visualized with a fluorescence microscope (Axiovert II with Axiocam HR, Zeiss, Göttingen, Germany), using the Axiovision program (Zeiss, Germany). For quantification of the fluorescence, biomass of the GFP-expressing *A.*

niger cultures was separated from the medium by filtration. 200 mg of the pellet were used to prepare cell extracts and th remaining mycelia were dried for three days at 60°C to determine the dry weight (DW). Production of GFP was quantified in the cell free extracts via fluorescence measurements (Luminescence Spectrometer LS50B, Perkin Elmer, Salem, USA). The fluorescence emission maximum of GFP was detected at 512 nm with an excitation at 475 nm, and the activity is given as specific fluorescence (INT $\mu g_{protein}^{-1}$). The TFH activity was determined with p-nitrophenyl-palmitate (pNPP) as substrate as described (Dresler et al. 2006).

Purification of affinity tagged reporter proteins

For purification of His_6-tagged proteins, approximately 200 mg WW of the over-expressing, recombinant *A. niger* strain was resuspended in 1 ml fluorescence buffer (see above) with 10 mM imidazole, and disrupted as described above. Subsequently, the soluble fraction of the extract was loaded onto Ni-NTA spin columns (Qiagen) and the His-tagged proteins were purified as described by the manufacturer. StrepII-tagged proteins were also purified from 200 mg WW, resuspended in 1 ml of buffer W (IBA GmbH, Göttingen, Germany), with the Strep-tag Starter Kit (IBA GmbH, Göttingen, Germany), following the instructions of the manufacturer.

Southern blot analysis of recombinant *A. niger* strains

Recombinant *A. niger* strains were grown under standard conditions. Cells were harvested by filtration, dried and 100 mg of mycelia were ground to powder in a liquid nitrogen-cooled mortar. Chromosomal DNA was prepared as described by Lee *et al.* 1988. For Southern blot analysis genomic DNA was digested by *Xho*I followed by electrophoresis, transfer onto a positively charged membrane (Roche, Basel) in 20 x SSC and UV cross-linkage. Blots were probed with a Dig-labelled fragment encoding

the recombinant gene of the expression plasmids. Prehybridization, hybridization to DIG-labelled riboprobes and membrane washing were conducted using the DIG luminescent Detection kit (Roche) according to the manufacturers instructions. *gfp* and *tfh* encoding fragments were detected with a DIG-labelled PCR fragment (DIG-PCR nucleotide mix, Roche) with primer pair *gfp-Fse*I-rev/*gfp-Cla*I-for and *tfh-Bsr*GI-*Age*I-rev/*tfh-Cla*I-for (Table S1), respectively.

RNA isolation and Northern detection

To compare and analyze the time course of *gfp* transcription, the recombinant *A. niger* strains were grown under standard conditions with 100 mM glucose as carbon source. Cultures were induced with 25 mM sucrose after 48 h and samples were taken at different time points after induction. Cells of the samples were harvested by filtration, dried and 100 mg of mycelia were ground to powder in liquid nitrogen-cooled mortar. Total RNA was isolated using the NucleoSpin® RNA Plant (Machery & Nagel, Düren, Germany) as described by the manufacturer. RNA concentration and quality were determined by measurement of A_{260} and A_{280}. Total cellular RNA (10 µg) was separated on formaldehyde agarose gels (1%), transferred overnight onto positively charged membranes (Roche, Mannheim, Germany) in 20 x SSC and UV cross-linked. Prehybridization, hybridization to DIG-labelled riboprobes and membrane washing were conducted using the DIG luminescent Detection kit (Roche) according to the manufacturers instructions. *gfp* transcripts were detected with a DIG-labelled PCR fragment (DIG-PCR nucleotide mix, Roche, Mannheim, Germany) applified with primer pair *gfp-Cla*I-for and *gfp-Fse*I-rev (Table S1), respectively.

Thin layer chromatography

Thin layer chromatography (TLC) analysis was done with 3 µl 1:3 diluted culture

broth (TLC, aluminium sheets 20S20 cm, silica gel 60 F254 with concentrating zone 20S2.5 cm, Merck, Germany). The carbohydrates were separated by three ascents in the solvent system ethylacetate/isopropanol/water 60:30:10 (v/v/v) at room temperature. Spots were visualized by dipping the plates into the detecting reagent consisting of 0.3% (w/v) N-(1-naphthyl)-ethylenediamine (Sigma-Aldrich, Munich, Germany), and 5% (v/v) concentrated sulfuric acid in methanol with the aid of a CAMAG Chromatogram Immersion Device III (Merck, Darmstadt, Germany), followed by heating in an oven at 120°C for 10 min. The sugars were visualized as dark spots. The marker contained 1 g/l fructose, glucose, sucrose, kestose and nystose.

Results

Identification and characterisation of the *sucA* promoter

For recombinant gene expression in *A. niger*, only very few well characterized promoters are available. To broaden the spectrum of inducible expression systems, we wanted to establish an alternative promoter system. Previous work, analyzing the expression and secretion of the endogenous β-fructofuranosidase (SucA) of the *A. niger* strain AB1.13 indicated that this enzyme is strongly secreted in the presence of sucrose (Boddy et al. 1993; Zuccaro et al. 2008). To test whether the *sucA* promoter is suitable for an inducible expression system in *A. niger*, a 1.2 kb segment, including the regulatory region of the *sucA* gene, was cloned into an integrative plasmid upstream of the reporter gene *gfp* of the jellyfish *Aequorea victoria* (Shimomura et al. 1962). The generated plasmid pARAn101 (P_{sucA}::*gfp*) was introduced into the *A. niger* strain AB1.13, and P_{sucA}-driven *gfp* expression of the *pyrG*$^{+}$ *A. niger* transformants was tested in the presence of different carbon sources: maltose, sucrose, xylose, fructose and glucose (100 mM). The spectrometrical analysis of the negative control

strains, wild-type strain AB1.13 and ARAn11 revealed very weak background fluorescence (Fig. 1, data not shown). A similar low fluorescence was also obtained with strain ARAn101, carrying the integrated P_{sucA}::*gfp* plasmid, when the fungus was grown with 100 mM maltose, xylose, fructose or glucose as single carbon source (Fig. 1A). In contrast, a strong induction of *gfp* expression was found when *A. niger* strain ARAn101 was grown with sucrose (Fig. 1A). In agreement with this observation, high levels of GFP were only detected on immunoblots of whole cell extracts of ARAn101, when the fungus was cultivated with sucrose. No GFP was detectable, when glucose, maltose or xylose were used as carbon source and only low amounts of GFP were visible in the presence of fructose (Fig. 1B). These results supported our previous assumption, that the *sucA* promoter is suitable for a new controllable *A. niger* expression system.

Analysis of the sucrose-driven expression of the *sucA* promoter

To further characterize the sucrose-inducible intracellular production of heterologous GFP in *A. niger*, we analyzed the efficiency of the *sucA* promoter at different growth temperatures (25°C, 30°C, 37°C and 42°C), at different pH values of the growth medium (2.5, 4.0, 5.5 and 7.0) and different degree of aeration (100 rpm, 140 rpm, 180 rpm and 220 rpm). Maximal GFP expression under the control of the *sucA* promoter was found when the recombinant fungus was cultivated at 37°C, 180 rpm at a medium pH of 5.5 (data not shown).

We also used the *A. niger* strain ARAn101 (P_{sucA}::*gfp*) to test whether sucrose-mediated expression of the *sucA* promoter is repressed in the presence of glucose, e.g. when sucrose is added as an inducer after cells were grown on glucose. Fortunately, no reduction of sucrose-dependent induction was detected in comparison to

ARAn101 grown with sucrose as single carbon source, and similar fluorescence levels were measured when both carbon sources were added (Fig. 2).

Next, we investigated P_{sucA}-driven GFP production over time after induction with 25 mM sucrose. Sucrose was added after 48 h growth in minimal medium under standard expression conditions with 100 mM glucose as single carbon source. GFP expression was visualized with a fluorescence microscope 0.5 to 4 and 24 hours after induction (Fig. 2). The microscopical analysis of ARAn101 (P_{sucA}::*gfp*) prior and 30 min after induction failed to detect fluorescence. In contrast, low levels of GFP were visible 1 h after addition of sucrose, and the amounts of the recombinant model protein increased strongly and continuously throughout the first hours of induction (Fig. 2).

To confirm and quantify P_{sucA}-driven GFP production by ARAn101, we performed a spectrometrical and an immunoblot analysis. As shown in Fig. 3A, no GFP protein is detectable in whole cell extracts prior to the addition of sucrose, and only a basal level of fluorescence, similar to the AB1.13 *A. niger* wild-type strain, was determined by the spectrometrical analysis. However, strain ARAn101 started to express GFP during the first hour after induction and accumulated increasing amounts of GFP (Fig. 3A,B). Fluorescence of the culture and the amount of GFP detected on western blots was maximal 9 h after induction and decreased when incubated overnight. Quantification of the *gfp* transcripts by Northern blotting and parallel analysis of the sucrose concentrations in the medium showed that the decrease of GFP activity resulted mainly from downregulation of the *sucA* promoter due to hydrolysis of the inducer to glucose and fructose (Fig. 3C,D). We also observed some degradation of GFP in cell extracts of the overnight culture (Fig. 3A). As proteolysis is significantly reduced in the presence of protease inhibitors (data not shown), it is likely that this proteolysis occurs after cell lysis through extracellular proteases produced during later growth

phases. Furthermore, *gfp* transcripts were not detectable in the presence of glucose in ARAn101 (Fig. 3C) and other recombinant strains (Fig. S1), harbouring multiple copies of the vector construct (for details see below). In summary, this demonstrated that the *sucA* promoter is tightly controlled and completely repressed in the absence of sucrose, and it is able to mediate fast and efficient production of heterologous, recombinant proteins after induction.

Expression vectors for the intracellular production of recombinant fusion proteins with small affinity tags in *A. niger*

A number of integrative expression vectors, using the newly identified sucrose-inducible *sucA* promoter and the highly efficient, constitutively expressed promoter of the pyruvate kinase gene *pki*A (de Graaff et al. 1992) were constructed based on ANIp8 (Storms et al. 2005) for the intracellular production and subsequent affinity purification of homologous and heterologous recombinant proteins by *A. niger*. All vectors contain an antibiotic resistance and a ColE1 *ori* sequence for the efficient maintenance and amplification in *E. coli*, and carry a *pyrG*$^{+}$ marker for the selection of chromosomally integrated plasmids in *A. niger* (Fig. 4). In order to obtain cloning flexibility and the opportunity to insert additional affinity-tags, protease sites or export sequences, the multiple cloning site was modified and extended to eight unique restriction sites (*Nhe*I, *Afl*II, *Cla*I, *Age*I, *Bsr*GI, *Bss*HII, *Eco*RI, *Fse*I) (Fig. 4, 5A). Codon-optimized sequences for a polyhistidine (His_6-) affinity tag (Porath et al. 1975) and a streptavidin (StrepII-) affinity tag (Schmidt et al. 1996) were cloned into the expression vector for the synthesis of N-terminal (Fig. 5B) or C-terminally (Fig. 5C) tagged proteins. This vector collection allows cloning of one restriction fragment, encoding the gene of interest, into all different expression vectors, and allows parallel

testing and selection of the most efficient production and purification system for the desired protein by *A. niger*.

Production of the His_6- and StrepII-tagged model proteins in *A. niger*

To test the production of affinity-tagged recombinant proteins, DNA fragments encoding the model proteins GFP or TFH were cloned in frame into the different expression vectors. After transformation into *A. niger* protoplasts, strains, harboring a chromosomally integrated copy of the expression plasmid, were isolated and production of the model proteins were tested by activity assays and western blotting with antibodies directed against the tag sequence or GFP. For this purpose, transformants were grown 48 h in minimal medium under standard expression conditions (37°C, pH 5.5) with 100 mM glucose for *pki*A promoter controlled expression or 100 mM sucrose to induce the protein expression through the *sucA* promoter. All vectors were tested with either TFH or GFP, and led to the production of the recombinant model protein in *A. niger* (Fig. 6). However, the amount of model proteins produced by a particular expression system was different between the individual transformants, most likely due to multiple insertions and different integration sites in the *A. niger* genome. In order to gain more information about the number of integrated expression plasmids we performed Southern blot experiments with chromosomal DNA of all recombinant *A. niger* strains. As shown in Fig. 7, 1-7 different fragments were detected with a *gfp* or *tfh* specific DNA probe. A band representing the integrated vector was identified in most strains, indicating that the expression strains contain tandem repeats of two or more integrated *gfp* and *tfh* expression plasmids. Notably, identical GFP fluorescence intensities were detected in expression constructs ARAn17 and ARAn101 with different numbers of integrated vector copies. This indicated that the

integration site has also an important influence on the expression of the integrated construct.

Purification of His_6-tagged model proteins

After detection, we also tested whether recombinant His_6-tagged protein can be isolated from the cell extract by an easy one-step purification procedure. *A. niger* strain ARAn18 (P_{pkiA}-*gfp*-His_6), containing a chromosomal insertion of vector pARAn18 that leads to the intracellular production of a C-terminally His_6-tagged GFP protein, was cultivated under standard conditions. The amount of GFP in the washing and elution fractions with increasing concentrations of imidazole were analyzed spectrophotometrically (Fig. 7A), by SDS-PAGE (Fig. 7B) and on immunoblots with antibodies directed against GFP (Fig. 7C) or the His_6-tag (Fig. 7D). The fluorescence of the column flowthrough was reduced to about 65% and the amount of bound GFP-His_6 was estimated to be about 17%. Washing with up to 50 mM imidazole eliminated unspecifically bound proteins, only 2% fluorescence remained in the second washing fraction in comparison to the cell extract. The highest amount of GFP was eluted at 100 mM imidazole with a purity of >95% (Fig. 7A-D, lanes E1). Approximately, 2 mg GFP-His_6 per g bio dry weight were purified. Some partially degradation of GFP-His_6 was seen on immunoblots with anti-GFP, but not with anti His_6-tag antibodies (Fig. 7C,D), indicating that degradation occurred from the C-terminal end. Also the TFH-His_6 protein expressed by *A. niger* strain ARAn15 (P_{pkiA}-*tfh*-His_6) was isolated by the same procedure. Active enzyme was eluted from the Ni-NTA affinity column at a concentration of 400 mM imidazole with a purity of >95% (data not shown).

StrepII-tagged GFP expressed by ARAn60 (P_{pkiA}-*gfp*-StrepII) and ARAn56 (P_{sucA}-StrepII-*gfp*) was also successfully purified from 30 ml cultures via a StrepTactin affinity column (2 mg g_{DW}^{-1}, data not shown). In general, up to 70% of the synthe-

sized StrepII- and up to 50% of the His_6-tagged model proteins were purified using standard protocols, independent whether the recombinant produced low or high amounts of the model proteins. This demonstrated that the tagged model proteins expressed by the new expression vector system in *A. niger* were easily detectable and could be purified in a one-step procedure in an active form.

Discussion

The limiting number of available vector systems and the increasing demand for molecular and biotechnological tools for protein and metabolite production by the filamentous fungi like *A. niger* intrigued us to develop new expression vectors. In this work a set of ten different integrative expression vectors for the intracellular synthesis of recombinant non-tagged and affinity-tagged target proteins were constructed and tested with two different heterologous model enzymes. The identical structure of the multiple cloning site with eight unique restriction sites offers cloning flexibility and allows parallel integration and fusion of the target genes to N- or C-terminal tag sequences. A highly efficient constitutive promoter of the *pki*A gene or a novel sucrose-inducible promoter of the endogenous β-fructofuranosidase SucA, can be used for expression of the target gene. The *pki*A promoter is expressed at high levels regardless of the carbon source, which permits high flexibility in the selection of growth conditions for maximal expression of the target protein.

Expression studies with integrative plasmids, in which expression is driven by the inducible *sucA* promoter, illustrated that maximal production of the model protein GFP was obtained at 37°C and pH 5.5, approximately 9 h after induction with sucrose. Subsequently, the transcription of target gene is downregulated due to hydrolysis of the inducer. We further showed, that the *sucA* promoter is not subject to repression by other carbon sources and can be activated by sucrose even in the

presence of glucose. Moreover, sucrose mediated activation of the promoter remained constant independent of the growth phase (A. Roth, unpublished results), suggesting that high induction levels can be maintained over longer time periods, when sucrose concentrations are kept constant in the growth medium. Since the expression of the target genes was completely repressed under non-inducing conditions (e.g. in the presence of glucose), but could be strongly induced, leading to a rapid and highly efficient protein production process, the P_{sucA}–driven expression vectors seem also very useful to synthesize heterologous proteins that are somehow unfavourable or deleterious for the fungus.

The expression vectors were used to express enhanced GFP (S65TGFP) or a hydrolase of *Thermobifida fusca* (TFH). Both selected heterologous model proteins could be easily detected and purified in a one-step procedure, when fused to the N- or C-terminal His_6 or StrepII affinity-tag sequence. However, gene expression by independent transformants of the identical integrative expression plasmid was highly different and varied up to 50-fold. Similar observations have been made with other integrative expression systems and have been attributed to different sites of integration and different copies of the integrated plasmids (Storms et al. 2005; Verdoes et al. 1994). We further plan to identify the integration sites of the plasmids which led to maximal expression of the model proteins, to use these chromosomal loci for the development of specific integrative expression plasmids to ensure high expression levels of the target proteins. Furthermore, we want to utilize the properties of the *sucA* promoter in combination with the coding sequence of the exoenzyme gene *sucA* for the development of expression vectors, which allow efficient secretion of recombinant *sucA* fusion proteins into the culture medium.

In conclusion, the new expression systems presented in this study offer three noteworthy features: (1) fast and efficient constitutive or inducible production, (2) easy detection, and (3) simple one-step purification of target proteins.

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References

Bakri Y, Al-Jazairi M, Al-Kayat G (2008) Xylanase production by a newly isolated *Aspergillus niger* SS7 in submerged culture. Pol J Microbiol 57:249-251

Boddy LM, Berges T, Barreau C, Vainstein MH, Dobson MJ, Ballance DJ, Peberdy JF (1993) Purification and characterisation of an *Aspergillus niger* invertase and its DNA sequence. Curr Genet 24:60-66

Bohlin C, Jönsson LJ, Roth R, van Zyl WH (2006) Heterologous expression of *Trametes versicolor* laccase in *Pichia pastoris* and *Aspergillus niger*. Appl Biochem Biotechnol 129:195-214

Bos CJ, Debets AJ, Swart K, Huybers A, Kobus G, Slakhorst SM (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr Genet 14:437-443

Chill L, Trinh L, Azadi P, Ishihara M, Sonon R, Karnaukhova E, Ophir Y, Golding B, Shiloach J (2009) Production, purification, and characterization of human α1 proteinase inhibitor from *Aspergillus niger*. Biotechnol Bioeng 102:828-844

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 20:448-455

Davies RW (1994) Heterologous gene expression and protein secretion in *Aspergillus*. Prog Ind Microbiol 29:527-560

de Graaff L, van den Broeck H, Visser J (1992) Isolation and characterization of the *Aspergillus niger* pyruvate kinase gene. Curr Genet 22:21-27

Dresler K, van den Heuvel J, Müller RJ, Deckwer WD (2006) Production of a recombinant polyester-cleaving hydrolase from *Thermobifida fusca* in *Escherichia coli*. Bioprocess Biosyst Eng 29:169-183

Fowler T, Berka RM, Ward M (1990) Regulation of the *glaA* gene of *Aspergillus niger*. Curr Genet 18:537-545

Ganzlin M, Rinas U (2008) In-depth analysis of the *Aspergillus niger* glucoamylase (*glaA*) promoter performance using high-throughput screening and controlled bioreactor cultivation techniques. J Biotechnol 135:266-271

Gordon CL, Archer DB, Jeenes DJ, Doonan JH, Wells B, Trinci AP, Robson GD (2000) A glucoamylase::GFP gene fusion to study protein secretion by individual hyphae of *Aspergillus niger*. J Microbiol Methods 42:39-48

Grote A, Hiller K, Scheer M, Munch R, Nortemann B, Hempel DC, Jahn D (2005) JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res 33:W526-531

Heim R, Cubitt AB, Tsien RY (1995) Improved green fluorescence. Nature 373:663-664

Karnaukhova E, Ophir Y, Trinh L, Dalal N, Punt PJ, Golding B, Shiloach J (2007) Expression of human α1-proteinase inhibitor in *Aspergillus niger*. Microb Cell Fact 6:34

Kraševec N, van de Hondel CA, Komel R (2000) Expression of human lymphotoxin-α in *Aspergillus niger*. Pflugers Arch 440:R83-85

Kusters-van Someren MA, Harmsen JA, Kester HC, Visser J (1991) Structure of the *Aspergillus niger pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet 20:293-299

Lee SB, Milgroom MG, Tayler JW (1988): A rapid, high-yield mini-prep method for isolation of total genomic DNA from fungi. Fungal Genetics Newsletter 35:23-24

Lichty JJ, Malecki JL, Agnew HD, Michelson-Horowitz DJ, Tan S (2005) Comparison of affinity tags for protein purification. Protein Expr Purif 41:98-105

Mattern IE, van Noort JM, van den Berg P, Archer DB, Roberts IN, van den Hondel CA (1992) Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. Mol Gen Genet 234:332-336

Pontecorvo G, Roper JA, Forbes E (1953) Genetic recombination without sexual reproduction in *Aspergillus niger*. J Gen Microbiol 8:198-210

Porath J, Carlsson J, Olsson I, Belfrage G (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. Nature 258:598-599

Punt PJ, van den Hondel CA (1992) Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. Methods Enzymol 216:447-457

Sambrook J, Russell DW 2001 Molecular cloning: a laboratory manual, 3rd edition, CSHL Press

Schmidt TG, Koepke J, Frank R, Skerra A (1996) Molecular interaction between the Strep-tag affinity peptide and its cognate target, streptavidin. J Mol Biol 255:753-766

Shah P, Bhavsar K, Soni SK, Khire JM (2009) Strain improvement and up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. J Ind Microbiol Biotechnol 36:373-380

Shimomura O, Johnson FH, Saiga Y (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. J Cell Comp Physiol 59:223-239

Storms R, Zheng Y, Li H, Sillaots S, Martinez-Perez A, Tsang A (2005) Plasmid vectors for protein production, gene expression and molecular manipulations in *Aspergillus niger*. Plasmid 53:191-204

van Hartingsveldt W, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel CA (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet 206:71-75

Verdoes JC, Punt PJ, van der Berg P, Debets F, Stouthamer AH, van den Hondel CA (1994) Characterization of an efficient gene cloning strategy for *Aspergillus niger* based on an autonomously replicating plasmid: cloning of the *nicB* gene of *A. niger*. Gene 146:159-165

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195-213

Ward M, Lin C, Victoria DC, Fox BP, Fox JA, Wong DL, Meerman HJ, Pucci JP, Fong RB, Heng MH, Tsurushita N, Gieswein C, Park M, Wang H (2004) Characterization of humanized antibodies secreted by *Aspergillus niger*. Appl Environ Microbiol 70:2567-2576

Wiebe MG, Karandikar A, Robson GD, Trinci AP, Candia JL, Trappe S, Wallis G, Rinas U, Derkx PM, Madrid SM, Sisniega H, Faus I, Montijn R, van den

Hondel CA, Punt PJ (2001) Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. Biotechnol Bioeng 76:164-174

Yang Y, Malten M, Grote A, Jahn D, Deckwer WD (2007) Codon optimized *Thermobifida fusca* hydrolase secreted by *Bacillus megaterium*. Biotechnol Bioeng 96:780-794

Zuccaro A, Götze S, Kneip S, Dersch P, Seibel J (2008) Tailor-made fructooligosaccharides by a combination of substrate and genetic engineering. Chembiochem 9:143-149

Figures

Fig. 1. Induction of the endogenous *sucA* promoter with different C-sources monitored through GFP. Strains AB1.13 (wild-type) and ARAn101 (P_{sucA}-*gfp*) were grown in liquid minimal medium with 100 mM of the different C-sources at standard conditions for two days. Specific fluorescence (INT $\mu g_{protein}^{-1}$) of the cultures was determined (**A**) and immunoblots were performed with whole cell extracts of ARAn101 (**B**) and AB1.13 (**C**) using antibodies directed against GFP. The fluorescence data represent the average ± SD of at least three different experiments each done in duplicate.

Fig. 2. P_{sucA}-mediated GFP expression in *A. niger. A. niger* strain ARAn101 (P_{sucA}-*gfp*) was cultivated for 48 h at standard conditions with 100 mM glucose in liquid minimal medium prior to induction with 100 mM sucrose. Samples were taken 0.5, 1, 2, 3, 4 and 24 hrs after induction of gene expression. Intracellular GFP production was visualized at two different exposure times (upper panels: phase contrast, lower panels: fluorescence emission at 512 nm).

Fig. 3. P_{sucA}-driven GFP expression after induction with sucrose. *A. niger* strain ARAn101 (P_{sucA}::*gfp*) was cultivated for 48 h in liquid minimal medium under standard conditions with 100 mM glucose prior to the induction with 25 mM sucrose. After induction, samples were taken at indicated time points and whole cell extracts as well as total RNA were prepared. Produced GFP in the cell extracts was visualized by immunoblotting, using an antibody directed against GFP (**A**), or was determined via its fluorescence by spectroscopical analysis and is given as specific fluorescence (INT $\mu g_{protein}^{-1}$) (**B**). The fluorescence data represent the average ± SD from at least three different experiments each done in duplicate. (**C**) Total RNA of the samples

were separated and Northern analysis was performed with a *gfp*-specific probe. The sizes of the RNA marker are indicated on the left, and the 18S rRNA of the loaded sample are shown below. (**D**) Thin layer chromatography was performed with culture supernatants. The carbohydrates were separated with the solvent system ethylacetate/isopropanol/water 60:30:10 (v/v/v) at room temperature and visualized as dark spots as described in Material and Methods. The sugars were visualized. The marker contained 1 g/l fructose, glucose, sucrose, kestose and nystose.

Fig. 4. The vector pARAn58 used the novel sucrose-inducible promoter of the *A. niger sucA* gene (P_{sucA}), and plasmid pARAn51 carries the constitutive *pki*A promoter (P_{pkiA}). Both vectors have identical multiple cloning sites, including a stop codon between the *Eco*RI and *Fse*I sites, followed by the terminator of the glucoamylase gene *gla*A (T_{gla}). The ColE1 *ori* sequence and the ampicillin resistance gene (*bla*) are used for the maintenance and replication in *E. coli*. The *pyrG* gene is required as selection marker for genome integrated plasmids in *A. niger*.

Fig. 5. Expression vector set for intracellular production of untagged and tagged proteins in *A. niger*. The constitutive *pki*A promoter (P_{pkiA}), given in black, or the newly identified inducible *sucA* promoter (P_{sucA}), given in grey, were introduced upstream of an extended multiple cloning site (MCS2). The MCS2 is followed by a stop codon and the *gla*A terminator (T_{gla}). All expression plasmids allow parallel cloning of the genes of interest into the MCS2. **A:** The basal expression vectors driven by P_{sucA} or P_{pkiA} with MCS2, containing eight unique restriction sites. **B:** The vector set with N-terminal His_6- and StrepII-tags. Three restriction sites *Nhe*I, *Afl*II and *Cla*I are located upstream of the N-terminal tags, that allow integration of export

sequences to permit protein secretion into the medium. **C:** The vector set with C-terminal His_6- and StrepII tags.

Fig. 6. Expression vectors tested with the model proteins GFP or TFH. *A. niger* transformants, harbouring the different integrated GFP or TFH expression plasmids, were grown 48 h under standard condition. Cell extracts were prepared and analyzed by western blotting with antibodies directed against StrepII- or the His_6-tag. The detected protein band of recombinant His_6- or StrepII-tagged GFP or TFH proteins is given in the boxes. Detection of GFP-fluorescence (> 3-fold higher than background) and TFH activity of the different newly constructed *A. niger* expression strains is also indicated. n. d.: not determined

Fig. 7. Southern blot analysis of recombinant overexpression strains. Chromosomal DNA of recombinant overexpression strain was digested with *Xho*I, electrophoretically separated, and transferred onto a positively charged membrane. Blots were probed with a Dig-labelled fragment encoding the *gfp* (A) or the *tfh* (B) gene of the expression plasmids. *Xho*I digested chromosomal DNA of the wild-type strain AB1.13, the *gfp* expression plasmid pARAn101, and the *tfh* expression plasmid pARAn15 were loaded as negative and positive controls. A molecular weight marker was loaded on the left.

Fig. 8. Purification of C-terminally His_6-tagged GFP expressed by the *A. niger* strain ARAn18 (P_{pkiA}-*gfp*-His_6). ARAn18 was grown 48 h under standard conditions with 100 mM glucose. Subsequently, cell extracts (CE) were prepared and applied onto a Ni-NTA spin column. The flow through (FT), the first washing step with 20 mM imidazole (W1), the second washing step with 50 mM imidazole (W2), and all elution

steps with 100 mM (E1), 400 mM (E2), 500 mM (E3) and 600 mM (E4) imidazole were collected. Specific fluorescence (INT $\mu g_{protein}^{-1}$) of the different samples was determined (A). Furthermore, samples were separated by 12% SDS-PAGE and stained by Coomassie Brilliant Blue (B) or transferred onto an Immobilon membrane and treated with antibodies directed against GFP (C) or the His_6-tag (D).

Supplementary material

Fig. S1. P_{sucA}-driven GFP expression prior and after induction with sucrose. *A. niger* strains ARAn17, ARAn55, ARAn56 and ARAn101 (P_{sucA}::*gfp*) were cultivated for 48 h in liquid minimal medium under standard conditions with 100 mM glucose prior to the induction with 25 mM sucrose. After induction, samples were taken at indicated time points and total RNA were prepared. Total RNA of the samples were separated and Northern analysis was performed with a GFP-specific probe. The sizes of the RNA marker are indicated on the left, and 18S rRNA of the loaded samples are shown below.

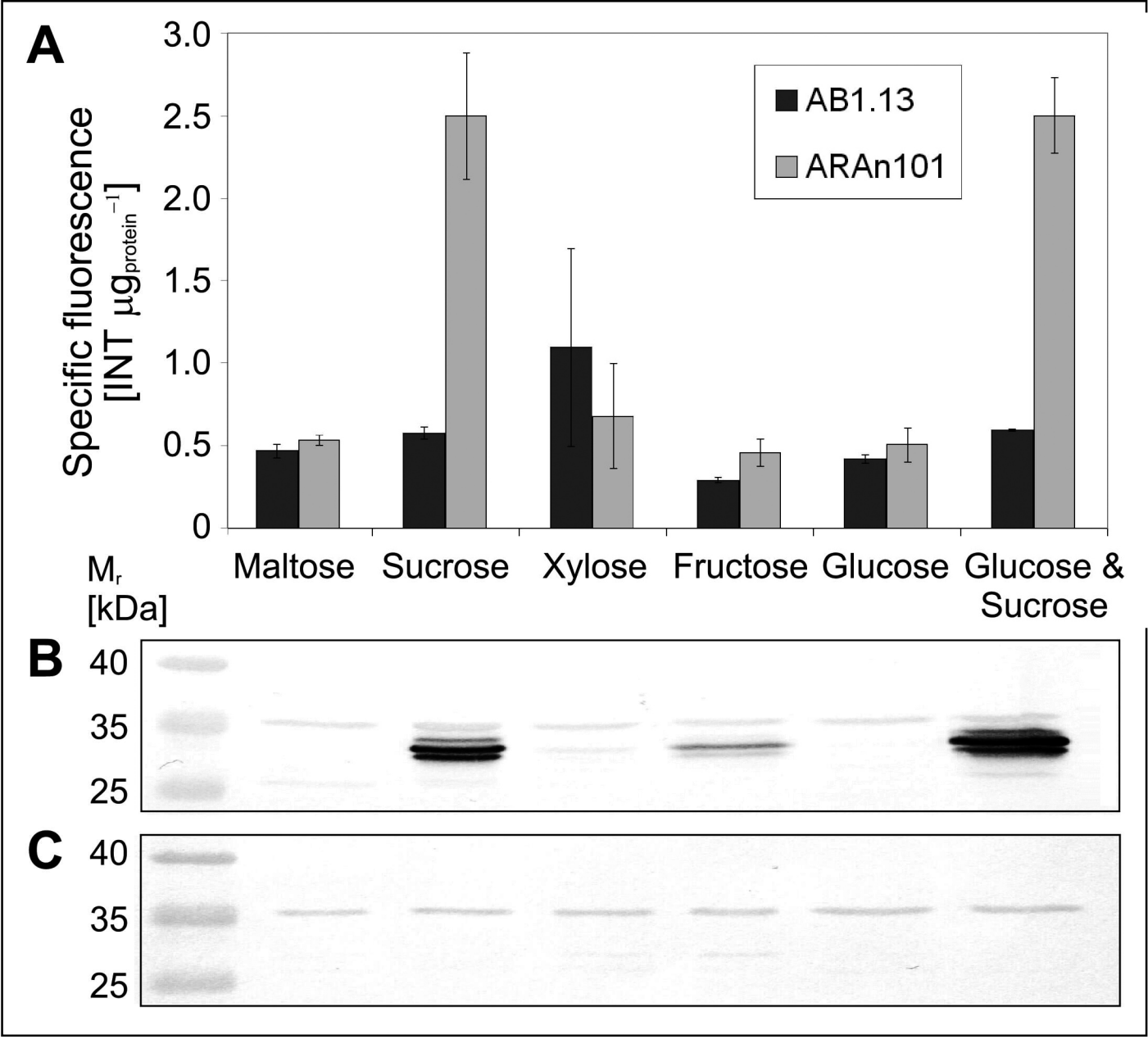


Fig. 1 Roth & Dersch 2009

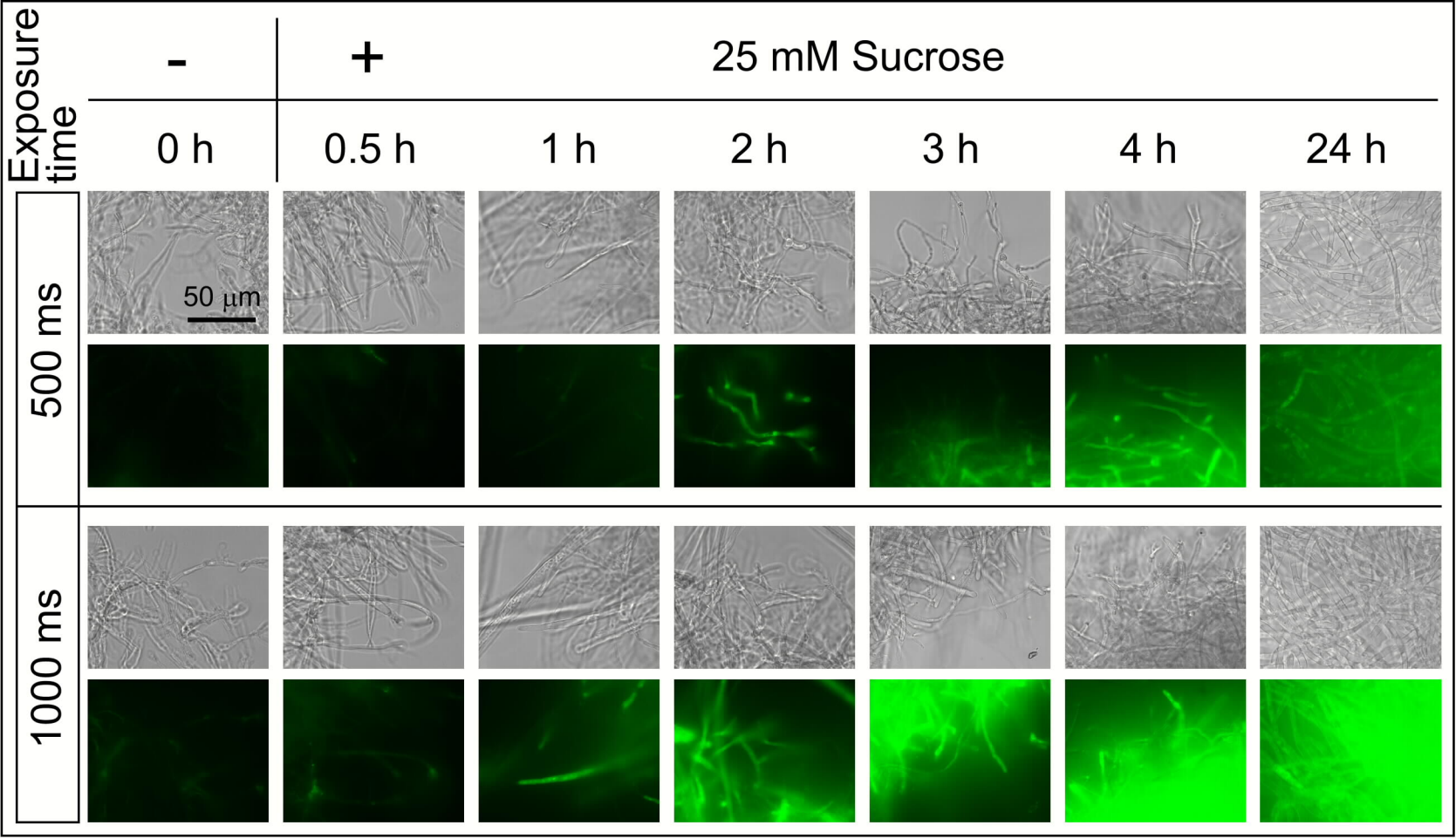


Fig. 2 Roth & Dersch 2009

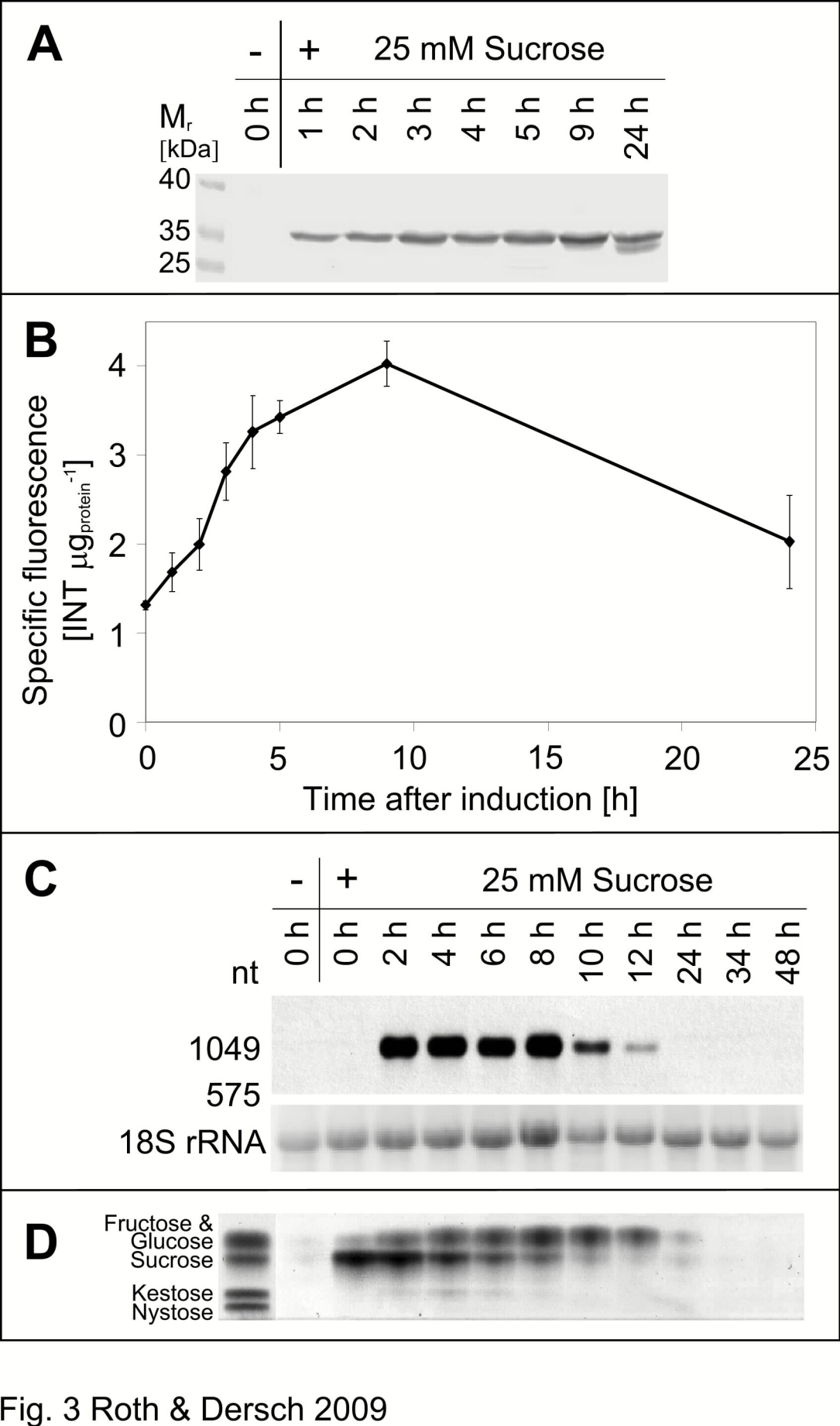


Fig. 3 Roth & Dersch 2009

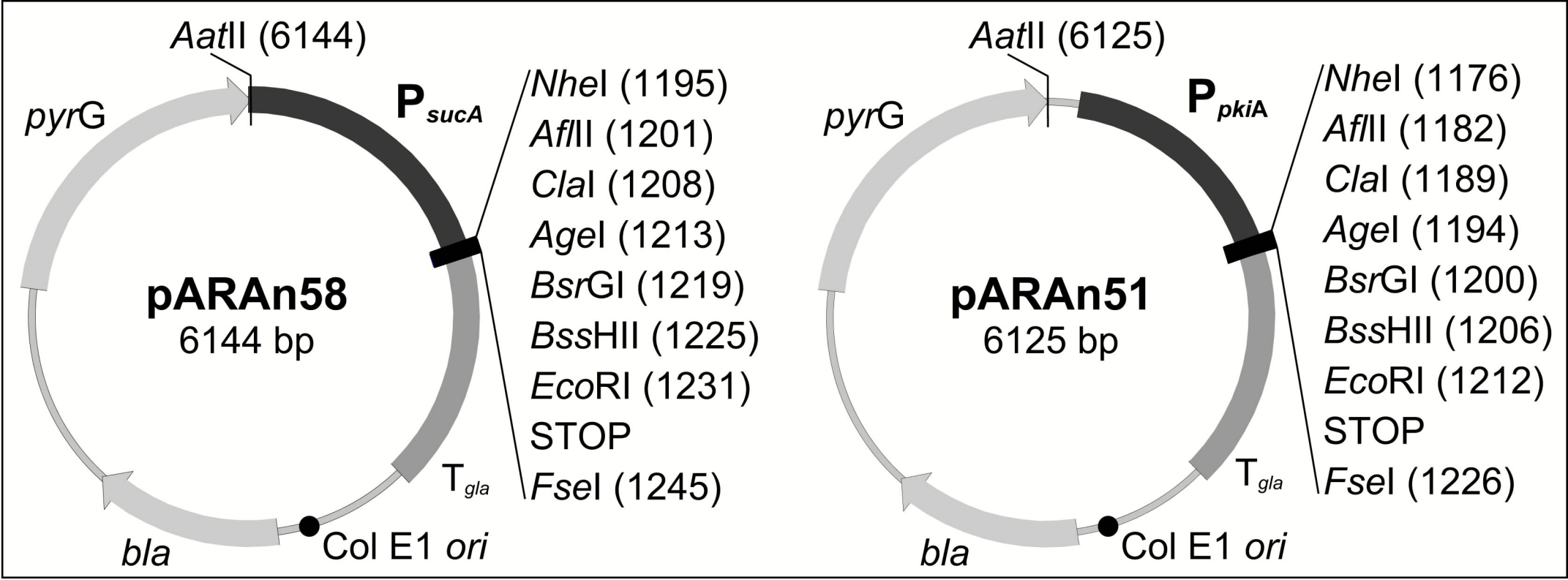


Fig. 4 Roth & Dersch 2009

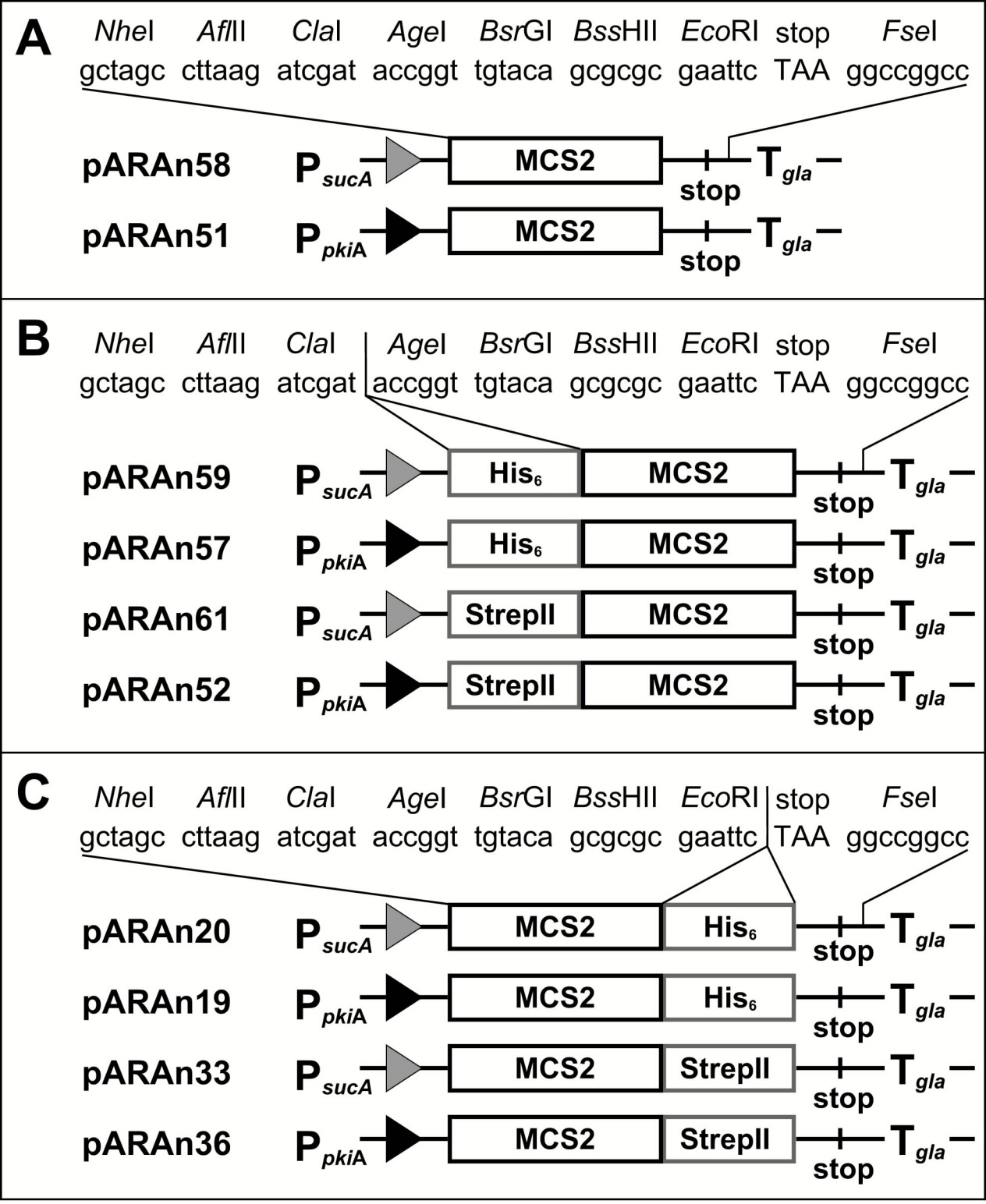


Fig. 5 Roth & Dersch 2009

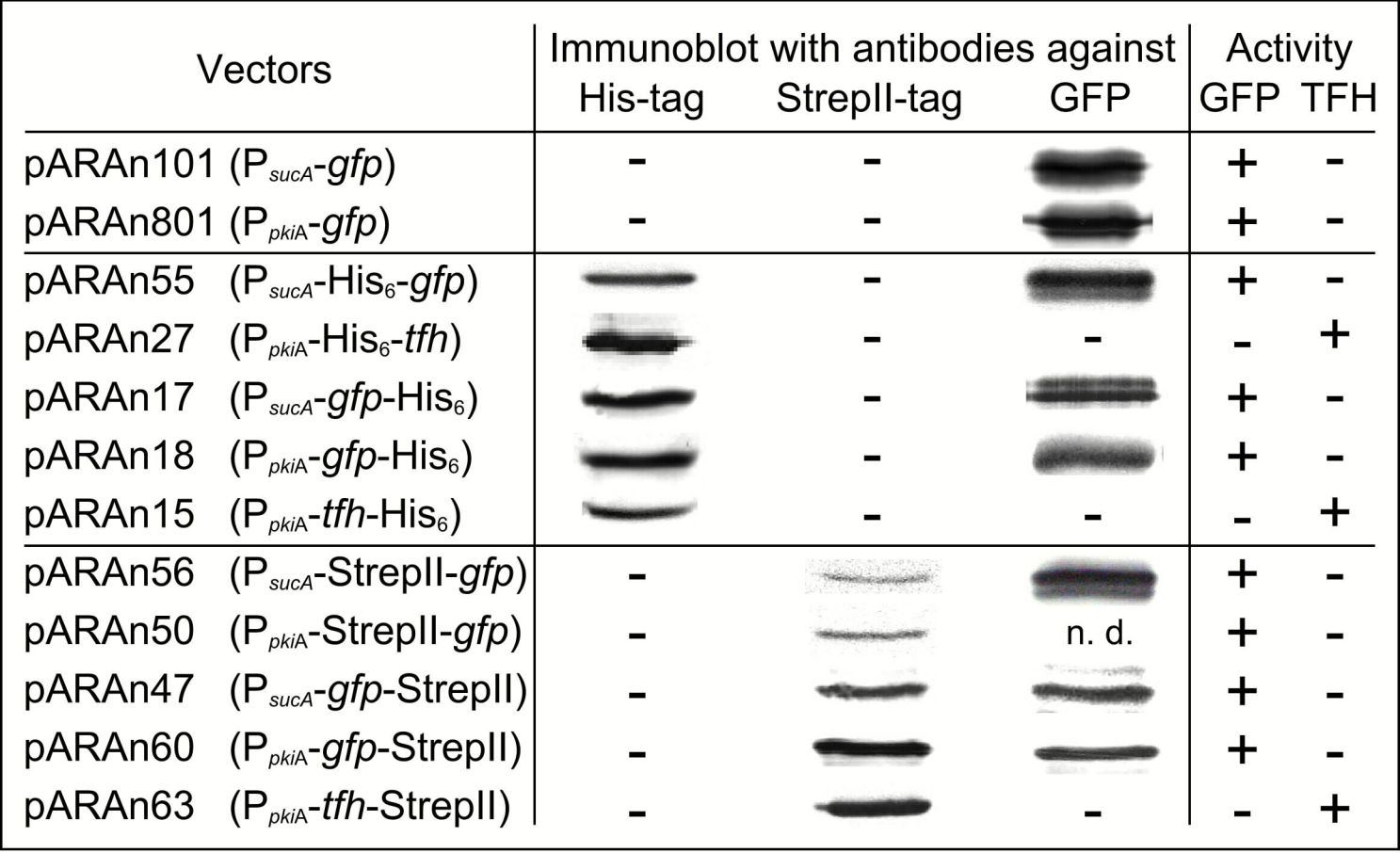


Fig. 6 Roth & Dersch 2009

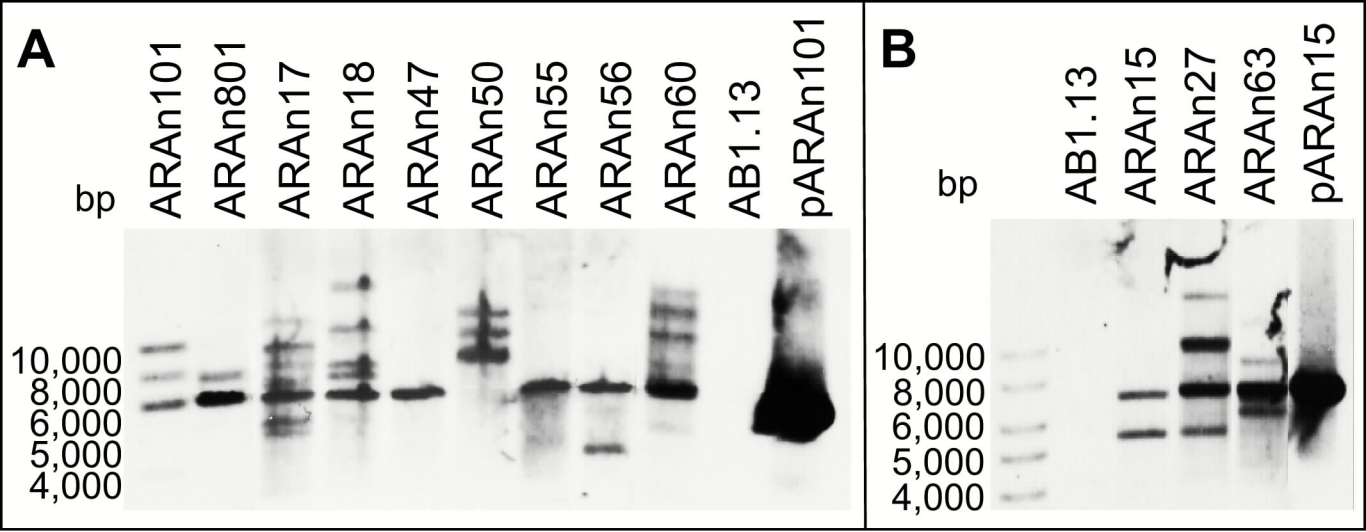


Fig. 7 Roth & Dersch 2009

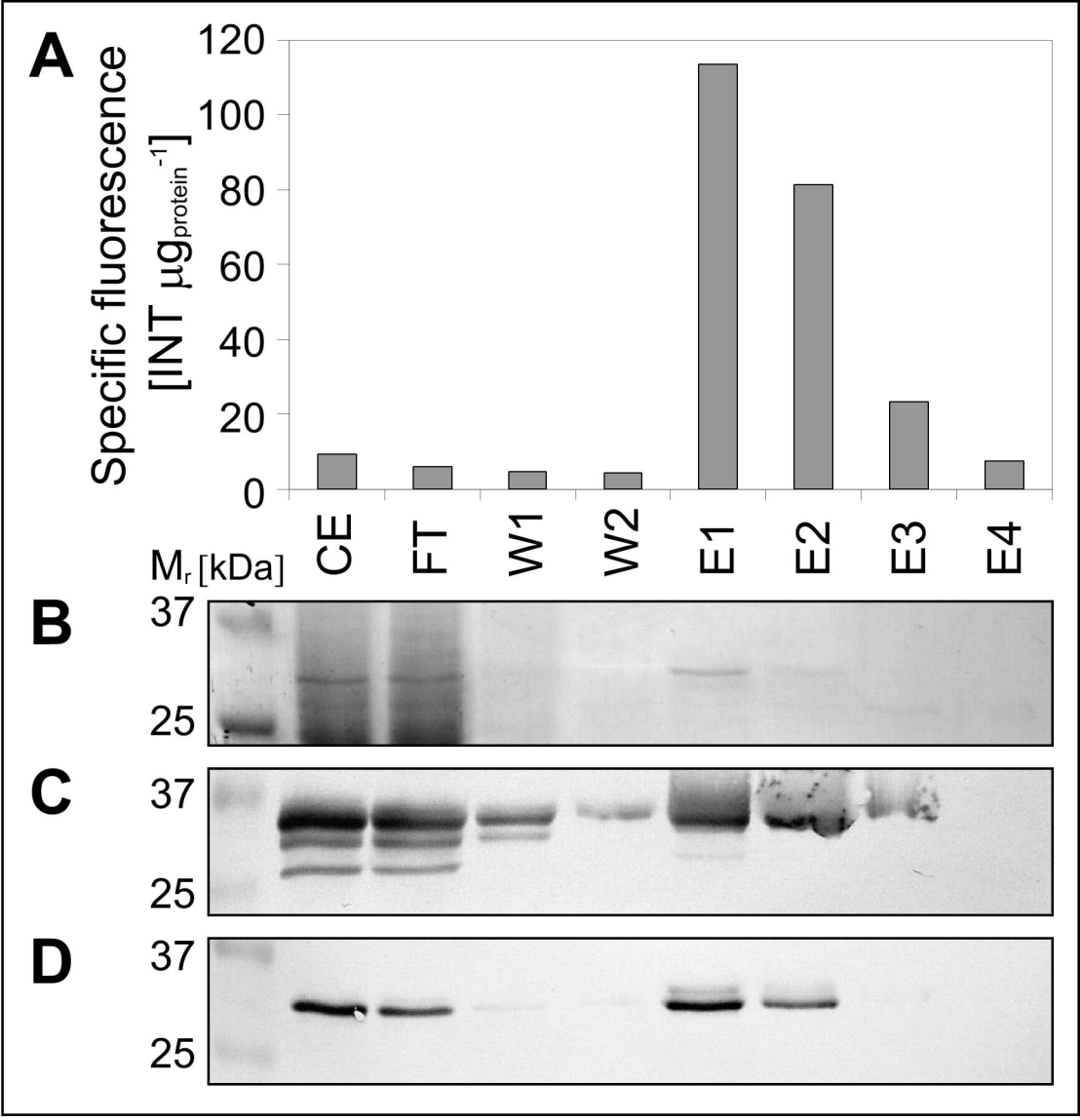


Fig. 8 Roth & Dersch 2009

Table 1. Strains and plasmid used in this study

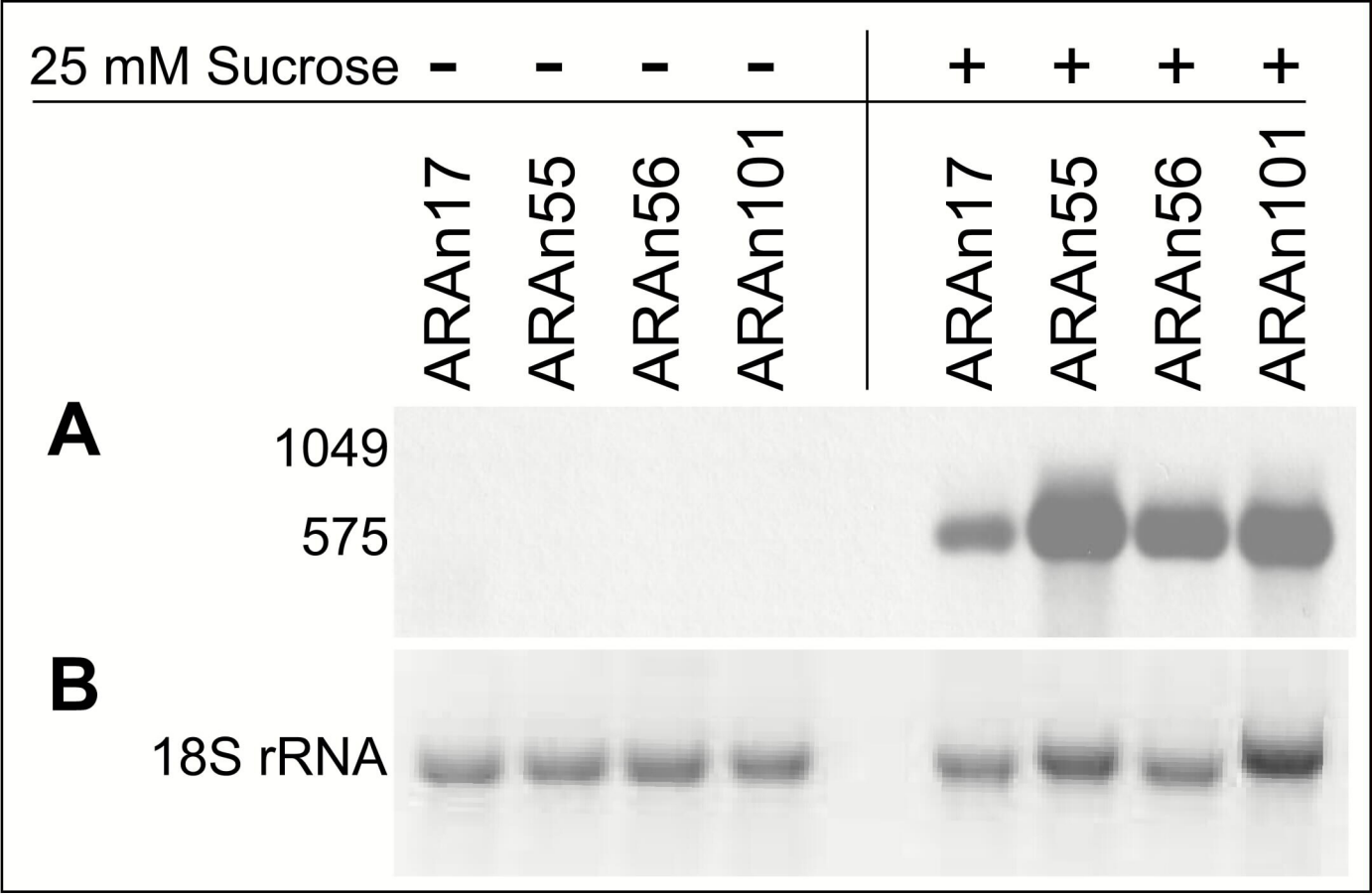
Name	Description	Reference or source
***A. niger* strains**		
AB1.13	Mutant of AB4.1, Δ*pepA*, Δ*prt-13* by UV irradiation	Mattern *et al.* (1992)
ARAn9	pARAn9 integrated into the genome of AB1.13	this work
ARAn11	pARAn11 integrated into the genome of AB1.13	this work
ARAn15	pARAn15 integrated into the genome of AB1.13	this work
ARAn17	pARAn17 integrated into the genome of AB1.13	this work
ARAn18	pARAn18 integrated into the genome of AB1.13	this work
ARAn19	pARAn19 integrated into the genome of AB1.13	this work
ARAn27	pARAn27 integrated into the genome of AB1.13	this work
ARAn47	pARAn47 integrated into the genome of AB1.13	this work
ARAn50	pARAn50 integrated into the genome of AB1.13	this work
ARAn55	pARAn55 integrated into the genome of AB1.13	this work
ARAn56	pARAn56 integrated into the genome of AB1.13	this work
ARAn60	pARAn60 integrated into the genome of AB1.13	this work
ARAn63	pARAn63 integrated into the genome of AB1.13	this work
ARAn101	pARAn101 integrated into the genome of AB1.13	this work
ARAn801	pARAn801 integrated into the genome of AB1.13	this work
***E. coli* strains**		
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara,leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ$^-$	Invitrogen
ER2925	*ara*-14 *leu*B6 *fhu*A31 *lac*Y1 *tsx*78 *gln*V44 *gal*K2 *gal*T22 *mcr*A *dcm*-6 *his*G4 *rfb*D1 R(*zgb*210::*Tn*10)*Tet*S *end*A1 *rps*L136 *dam*13::*Tn*9 *xyl*A-5 *mtl*-1 *thi*-1 *mcr*B1 *hsd*R2	New England Biolabs (NEB)
Top10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 *gal*U *gal*K Δ(*ara-leu*)7697 *rps*L (StrR) endA1 *nup*G	Invitrogen
Plasmids		
Expression vectors		
ANEp8	pUC18-derivative, *ARSAMA*1, *pyr*G, P_{pkiA}, T_{gla}, Ap^R	Storms et al. (2005)
ANIp8	ANEp8-derivative Δ*ARSAMA*1	this work
pARAn1	pANIp8-derivative, P_{suc1}-MCS1	this work
pARAn8	pANIp8-derivative, P_{pkiA}-MCS1	this work
pARAn9	pANIp8-derivative, P_{pkiA}-MCS2	this work
pARAn11	pANIp8-derivative, P_{suc1}-MCS2	this work
pARAn19	pANIp8-derivative, P_{pkiA}-MCS2-His_6	this work
pARAn20	pANIp8-derivative, P_{suc1}-MCS2-His_6	this work
pARAn31	pANIp8-derivative, P_{pkiA}-His_6-MCS2	this work
pARAn32	pANIp8-derivative, P_{suc1}-His_6-MCS2	this work
pARAn33	pANIp8-derivative, P_{suc1}-MCS2-StrepII	this work
pARAn34	pANIp8-derivative, P_{suc1}-StrepII-MCS2	this work
pARAn36	pANIp8-derivative, P_{pkiA}-MCS2-StrepII	this work
pARAn37	pANIp8-derivative, P_{pkiA}-StrepII-MCS2	this work
pARAn51	pANIp8-derivative, P_{pkiA}-MCS2-STOP, Acc. No. FJ868796	this work
pARAn52	pANIp8-derivative, P_{pkiA}-StrepII-MCS2-STOP	this work
pARAn57	pANIp8-derivative, P_{pkiA}-His_6-MCS2-STOP	this work
pARAn58	pANIp8-derivative, P_{suc1}-MCS-STOP, Acc. No. FJ868795	this work
pARAn59	pANIp8-derivative, P_{suc1}-His_6-MCS2-STOP	this work
pARAn61	pANIp8-derivative, P_{suc1}-StrepII-MCS2-STOP	this work
gfp and *tfh*		

expression vectors		
pARAn15	pARAn9, P_{pkiA}::*tfh*-His_6	this work
pARAn16	pARAn11, P_{suc1}::*tfh*-His_6	this work
pARAn17	pARAn11, P_{suc1}::*gfp*-His_6	this work
pARAn18	pARAn11, P_{pkiA}::*gfp*-His_6	this work
pARAn27	pARAn9, P_{pkiA}::His_6-*tfh*	this work
pARAn29	pARAn11, P_{suc1}::His_6-*tfh*	this work
pARAn47	pARAn33, P_{suc1}::*gfp*-StrepII	this work
pARAn49	pARAn9, P_{pkiA}::*gfp*-STOP	this work
pARAn50	pARAn37, P_{pkiA}::StrepII-*gfp*-STOP	this work
pARAn53	pARAn31, P_{pkiA}::His_6-*gfp*-STOP	this work
pARAn54	pARAn11, P_{suc1}::*gfp*-STOP	this work
pARAn55	pARAn32, P_{suc1}::His_6-*gfp*-STOP	this work
pARAn56	pARAn34, P_{suc1}::StrepII-*gfp*-STOP	this work
pARAn60	pARAn36, P_{pkiA}::*gfp*-StrepII	this work
pARAn63	pARAn36, P_{pkiA}::*tfh*-StrepII	this work
pARAn101	pARAn1, P_{suc1}::*gfp*	this work
pARAn801	pARAn8, P_{pkiA}::*gfp*	this work

Table 2. Oligonucleotides used in this study

Name	**Description**
MCS oligos	
MCS-*Nco*I-for (MCS1)	gccaggccatgggctagcttaagtttaaacgatatcgatggcgccgggcccaggccggccccatggagacgg
MCS-*Nco*I-rev (MCS1)	ccgtctccatggggccggcctgggcccggcgccatcgatatcgtttaaacttaagctagcccatggcctggc
new-MCS-for (MCS2)	tatagctagccttaagatcgataccggttgtacagcgcgcgaattcggccggcctata
new-MCS-rev (MCS2)	tataggccggccgaattcgcgcgctgtacaaccggtatcgatcttaaggctagctata
P_{suc1} primers	
P_{suc1}-*Aat*II (F)	cacgacgtcagtagtgatatgtcggctctagttccttgctgtaag
P_{suc1}-*Nhe*I (R)	gcttcatggctagcaatagcagaggaggggacactc
***gfp* primers**	
*gfp-Bss*HII-*Eco*RI-for (C-terminal His tag)	tatagcgcgcgaattcatggtgagcaagggcgaggagc
*gfp-Eco*RI-H-*Fse*I-rev (C-terminal His tag)	cggcatggacgagctgtacaaggaattccaccaccaccaccaccactaaggccggcctata
*gfp-Cla*I-H-*Age*I-for (N-terminal His tag)	tataatcgatatgcaccaccaccaccaccacaccggtgtgagcaagggcgaggagc
*gfp-Eco*RI-Stop-*Fse*I-rev	tataggccggccttagaattccttgtacagctcgtccatgccg
PCR-*gfp-Afl*II-for	gggagcttaagcatggtgagcaagggcgagg
PCR-*gfp-Cla*I-rev	cgcaggatcgatttacttgtacagctcgtcc
*gfp-Fse*I-rev	atataggccggccttacttgtacagctcgtccatgccg
*gfp-Cla*I-for	tatatatcgatatggtgagcaagggcgagg
***tfh* primers**	
*tfh-Bss*HII-*Eco*RI-for (C-terminal His tag)	tatagcgcgcgaattcatggccgccaacccctacgagcg
*tfh-Eco*RI-His-*Fse*I-rev (C-terminal His tag)	tataggccggccctagtggtggtggtggtggaattcgaacgggcaggtggagc
*tfh-Cla*I-*Age*I-His-for (N-terminal His tag)	tataatcgatatgcaccaccaccaccaccacaccggtgccgccaacccctacgagcg
*tfh-Bsr*GI-*Age*I-rev (N-terminal His tag)	tatatgtacaaccggtctagaacgggcaggtggag
*tfh-Cla*I-for	tataatcgatatggccgccaacccctacgagcg
StrepII-tag oligos	
C-Strep-for	aattcaactggtcccacccccagttcgagaagtaatctagaggccgg
C-Strep-rev	cctctagattacttctcgaactgggggtgggaccagttg
N-Strep-for	cgattctagaatgaactggtcccacccccagttcgagaaga
N-Strep-rev	ccggtcttctcgaactgggggtgggaccagttcattctagaat
sequencing primers	
P_{pkiA}(F)	gccagctctcaacccgcggggaaccgaccg
seq-P_{suc1}-for	cgtcccgcagagcatacg
seq-promoter-for	gaggaaggctggaaagcttacg
seq-P_{suc1}-F	cacccctgcatcaacagaagc
seq-ANIp7-1-for	atccaagcacgtaattccatagc
seq-ANIp7-2-rev	atagccaaacctcatctgcacg
seq-ANIp7-3-for	gacggagtgtcggtgagtg

seq-ANIp7-MCS-rev	cactactattatgcacacccactac
seq-ANIp7-6-rev	ccgcctcgactggatcg
seq-ANIp7-7-for	gcatccgcttacagacaagc
seq-ANIp7-8-rev	gtcgcgagggattggagc
seq-ANIp7-9-for	gtcaggcaactatggatgaacg
seq-ANIp7-10-rev	cgactacatttacatcaaatcctttgc



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