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The PARS sequence increase the efficiency of stable *Pichia pastoris* transformation

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ABSTRACT

The methylotrophic yeast *Pichia pastoris* is a popular host for recombinant expression of proteins. Plasmids containing the *Pichia* autonomously replicating sequence (PARS) transform *P. pastoris* with higher efficiency than linear DNA equipped with termini designed for homologous recombination. Moreover, PARS containing constructs provide higher protein yields. Unfortunately, these autonomous plasmids are inherently unstable and the preferred method of *P. pastoris* transformation is therefore stable integration in the genome by homologous recombination. In the present study we report that a novel combination of PARS and linearization of plasmids for *P. pastoris* transformation serves to significantly increase the transformation efficiency. Moreover, it is demonstrated that the constructs do not re-circularize but integrate stably into the *P. pastoris* genome.

1. Introduction

Autonomously replicating sequences (ARS) were first identified in baker's yeast (*Saccharomyses cerevisiae*) as sequences from the yeast genome which enable plasmids to replicate autonomously (Struhl et al., 1979). Genomic ARS sequences are believed to serve as origins of DNA replication during mitosis (Huberman et al., 1988). A functionally similar sequence (PARS) has been identified in the popular recombinant expression host *Pichia pastoris* (Cregg et al., 1985). *S. cerevisiae* and *P. pastoris* are transformed with high efficiency by circular plasmids containing ARS or PARS respectively (Cregg et al., 1985, Struhl et al., 1979). Unfortunately, autonomous *P. pastoris* plasmids have a high propensity to integrate in the genome if they contain segments with homology to the *P. pastoris* genome, e.g. endogenous promoter or terminator sequences. A plasmid with more than 500 bp homology will integrate within the first 100 generations after transformation (Higgins and Cregg, 1998). Such uncontrolled integration events are undesired because they convert the originally isolated clones with autonomous plasmids into a heterogeneous mixture of cells with various patterns of construct integration. Transgene expression is associated with a considerable metabolic burden (Görgens et al., 2001). It should therefore be expected that clones where only the selectable marker is expressed will have the competitive advantage and gradually become dominant thus compromising expression levels.

There are few publications where the PARS sequence has been used in constructs for routine recombinant expression. Of the 42 expression studies listed by Macauley-Patrick et al. (2005) only one (Aoki et al. (2003)) used a vector with the PARS sequence. Steinle et al. (2010) used *His*4 complementation to stabilize autonomous plasmids for the expression of cyanophycin synthetases. In addition, the plasmids harbored zeocin resistance which was used to evaluate the stability of the plasmids. While this method does not distinguish between integrated and autonomous plasmids, the authors did show that between 12 and 15% of cells in a flask culture would lose zeocin resistance in the absence of selection. *His*4 complementation retained zeocin resistance in 98% of the cells cultivated in the absence of zeocin. Hong et al. (2006) evaluated the potential of pGAPz-B with PARS inserted at the *Bam*HI site as an autonomous plasmid vector. The stability of this vector was only 35% after 24 h or 10 generations without selection. The authors achieved improved yields of β-galactosidase compared to a stable transformant control when their autonomous plasmid clone was grown under zeocin selection. The continued presence of autonomous plasmids was confirmed by re-transformation of *E. coli* but the percentage of cells which retained autonomous plasmids was not determined. Lueking et al. (2000) reported shuttling 29 PARS plasmids from *E. coli* to *P. pastoris* and back but did not quantify their stability.

The most widely used vectors are the pPIC and pGAP series which do not contain the PARS sequence (Macauley-Patrick et al., 2005). These are "ends in" vectors and constructs in this type of vector are linearized prior to *P. pastoris* transformation to promote homologous recombination between promoter regions on the plasmids and in the genome.

Here we show that linearized plasmids containing the PARS sequence retain high transformation efficiency and integrate into the genome by recombination at the linearization site. This means that transformation efficiencies are significantly increased without

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Table 1
PCR primers used for cloning and control PCR reactions.

Primer name	Primer sequence
PARS1 Fus Fw	5′GCATGAGATCAGATCCAATTAATATTTACTTATTTTGGT3′
PARS1 Fus Rv	5′TCTACAAAAAAGATCGATAAGCTGGGGGAACATT3′
Int TP Fw	5′ATTGAACAACTATTTAAAAATGGCATCTTTAGTCAAAAAGG3′
TP His *Xba*I Rv	5′GATGAGTTTTTGTTCTAGTTAATGATGATGATGATGATGGTCGACGAATTCTGG3′
pUC-GAP Fw	5′GCAGAGCGAGGTATGTAGGC3′
pUC-GAP Rv2	5′CCGTCAACGGTCTTTTGAGT3′
GAPflank Fw	5′CGATCAATGAAATCCATCAAGA3′
TPint Rv	5′CGGTACCTTCCATCGTCATT3′

compromising the benefits of stable chromosomal integration. The functionality of the resulting transformants was confirmed by expression of the chromogenic reporter protein TinselPurple.

2. Materials and methods

2.1. Molecular cloning

The PARS1 sequence was amplified from pPICHOLI (MoBiTec, Germany) using primers PARS1 Fus Fw/Rv (Table 1). The PARS1 amplicon was inserted in pGAPzαB (ThermoFisher scientific, USA) at the *Bgl*I site. This was achieved by In-Fusion cloning (Clontec, USA) and resulted in pGAPzαB-PARS.

The TinselPurple coding sequence was amplified from plasmid CPB-38-902 (DNA2.0, USA) with primers Int TP Fw and TP His *Xba*I Rv (Table 1). The TinselPuple amplicon was inserted into pGAPzαB, linearized with *Bst*BI and *Xba*I, by In-Fusion cloning (Clontec, USA) resulting in pGAPzαB-intTP (Fig. 1, left).

A fragment containing the TinselPurple coding sequence was subcloned from pGAPzαB-intTP to pGAPzαB-PARS after *Avr*II and *Bam*HI digestion, gel purification of the relevant fragments followed by ligation with T4 DNA ligase. The final plasmid was named pGAPzαB-PARS-intTP (Fig. 1, right).

2.2. Pichia transformation

Competent cells were prepared by a modified condensed protocol (Lin-Cereghino et al., 2005). Briefly, 20 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) in a 250 mL baffled flask was inoculated with *P. pastoris* KM71H cell mass transferred from a plate culture with a 3 mm inoculation loop. The culture was incubated at 27 °C with 230 rpm shaking overnight. A further 80 mL of YPD was added after 18 h where after the incubation was proceeded for 4 h. At this point, a dilution series for OD_{600} measurement was prepared and the cells were harvested by centrifugation for 10 min at 1500 ×*g*. Typical OD_{600} values ranged from 6 to 10.

The cells were washed three times with 50 mL of sterile demineralized water at the above mentioned centrifuge settings. The washed cells were re-suspended in 25 mL buffer SED (1 M sorbitol, 50 mM Tris HCl, 25 mM EDTA, 10 mM DTT pH 7.5) and incubated at ambient temperature for 18 min. The SED treated cells were spun down at 1500 ×*g* at 4 °C for 5 min and washed twice with 25 mL ice cold 1 M sorbitol at the same centrifuge settings. Finally, the cells were re-suspended in ice cold 1 M sorbitol to an OD_{600} of 100 based on the initial OD measurement.

The cells were kept on ice and used for transformation immediately.

2.3. Preparation of linearized DNA

Plasmid midi preps were prepared using the Nucleobond Xtra kit (Macherey-Nagel) according to the manufactures instructions. Restriction digests with *Avr*II and *Bsp*HI were carried out over night at 37 °C in 1 × cutsmart buffer, with 0.1 μ of enzyme per μg plasmid at a plasmid concentration of 0.5 μg/μL (all reagents from New England Biolabs, USA). The linearized plasmids were agarose gel purified and the concentration was measured on a Nanodrop spectrophotometer.

2.4. Transformation

The desired DNA amount was mixed with 400 μL of competent cells in a 0.2 cm Gene Pulser cuvette (BIO-RAD) and incubated on ice for 30 min before electroporation. The pulse settings were 1.8 kV, 200 Ω and 25 μF. The cells were transferred to a 1.5 mL centrifuge tube with the help on 1 mL 1 M sorbitol and incubated at 28 °C o/n before plating on YPD agar with 100 μg/mL zeocin. Plates were incubated at 28 °C for two days at which point the colonies were counted. Colonies selected for DNA purification were propagated in liquid YPD and *P. pastoris* total DNA was purified according to (Lõoke et al., 2011).

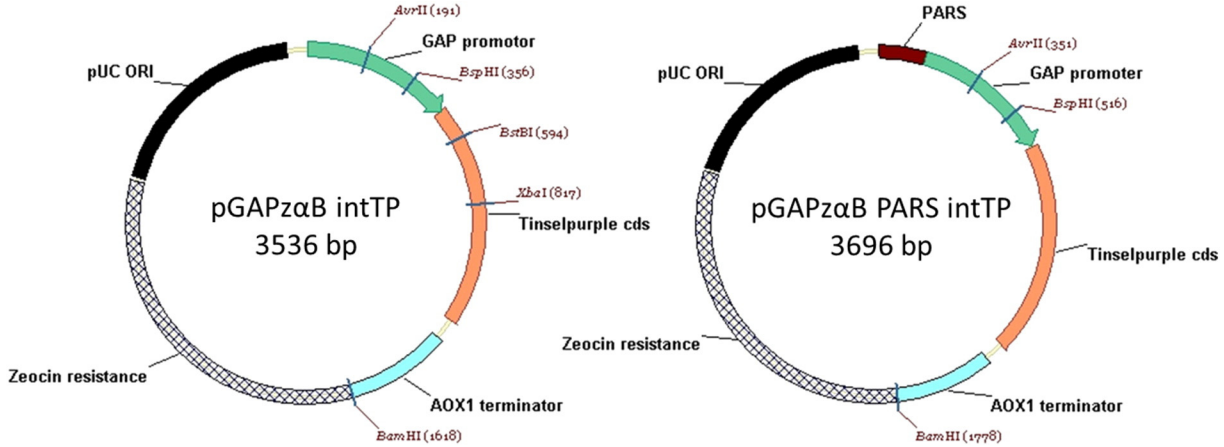

Fig. 1. The two constructs used for *P. pastoris* transformation.