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Tools and Techniques

Cre-*lox*P-based system for removal and reuse of selection markers in *Ashbya gossypii* targeted engineering

Tatiana Q. Aguiar [1], Cláudia Dinis [1], Lucília Domingues *

CEB – Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal

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ABSTRACT

The filamentous ascomycete *Ashbya gossypii* is amenable to genetic manipulation and is an excellent model system for studying eukaryotic cell biology. However, the number of selection markers in current use for both targeted gene integration and disruption in this fungus are very limited. Therefore, the Cre-*lox*P recombination system was adapted for use in *A. gossypii* and its effectiveness in recycling marker genes was demonstrated by constructing both single and double deleted *Agura3* and *Agade1* auxotrophic strains free of exogenous markers. In spite of its wide use, this is the first report in which the Cre-*lox*P system was applied to *A. gossypii*, opening new perspectives for targeted engineering of this fungus with several promising biotechnological applications.

1. Introduction

The Cre-*lox*P recombination system of the bacteriophage P1 has been shown to mediate efficient recombination between directly repeated *lox*P sites flanking selectable markers in several organisms, resulting in the excision of marker genes (Sternberg and Hamilton, 1981; Sauer and Henderson, 1988; Orban et al., 1992; Zhang et al., 2009). Cre-*lox*P-based systems have been widely used to study gene function and to remove selection markers in fungi, among which *Saccharomyces cerevisiae* (Sauer, 1987; Güldener et al., 1996), *Kluyveromyces lactis* (Steensma and Ter Linde, 2001), *Kluyveromyces marxianus* (Ribeiro et al., 2007), *Schizosaccharomyces pombe* (Iwaki and Takegawa, 2004), *Yarrowia lipolytica* (Fickers et al., 2003), *Hansenula polymorpha* (Qian et al., 2009), *Cryptococcus neoformans* (Patel et al., 2010), *Aspergillus nidulans* (Forment et al., 2006), *Trichoderma reesei* (Steiger et al., 2011), *Neotyphodium coenophialum*, *N. uncinatum* and *Epichloë festucae* (Florea et al., 2009).

Ashbya gossypii (syn. *Eremothecium gossypii*) is a flavinogenic filamentous ascomycete (Ashby and Nowell, 1926; Kurtzman, 1995) used for the industrial production of riboflavin (Stahmann et al., 2000), which shares remarkable genomic similarities with the budding yeast *S. cerevisiae* (Brachat et al., 2003; Dietrich et al., 2004), being therefore considered an excellent model to study filamentous growth (Wendland and Walther, 2005; Schmitz and Philippsen, 2011). The high degree of gene homology and gene order conservation (synteny) between the *A. gossypii* genome and the *S. cerevisiae* genome have facilitated the assignment of potential functions to *A. gossypii* genes (Brachat et al., 2003; Dietrich et al., 2004). Moreover, other *A. gossypii* features have made the genetic manipulation of this fungus to be quite straightforward when comparing to other filamentous fungi, among which: highly efficient homologous recombination, which allows the use of PCR-based gene targeting tools (Steiner et al., 1995; Wendland et al., 2000); haploid nuclei and unicucleated spores (Steiner et al., 1995), which facilitate the isolation of stable homokaryotic strains; and ability to freely replicate plasmids containing *S. cerevisiae* autonomously replicating sequences (ARS) (Wright and Philippsen, 1991) and to regulate gene expression using *S. cerevisiae* promoters (Wendland et al., 2000; Kaufmann, 2009; Ribeiro et al., 2010; Magalhães et al., 2014), making possible the use of several tools already available to manipulate *S. cerevisiae*.

Although a wide range of molecular tools exist to genetically engineer *A. gossypii*, the number of selection markers in current use is limited. The absence of a known sexual cycle in *A. gossypii* has hindered the use of classical genetics approaches in this fungus (Wendland et al., 2011). Thus, the molecular characterization of *A. gossypii* genes has relied on reverse genetics approaches. However, only four marker genes are available for PCR-based gene targeting in *A. gossypii*, which has hampered the disruption and/or integration of multiple genes in the same strains. The existent markers are: (1) the *S. cerevisiae LEU2* gene fused with its own promoter and terminator (Alberti-Segui et al., 2001), which can only be used as auxotrophic marker for gene manipulations in stable *Agleu2*

* Corresponding author. Fax: +351 253 604429.
E-mail address: luciliad@deb.uminho.pt (L. Domingues).
[1] These authors contributed equally to this work.

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strains; (2) the *Escherichia coli kan*^{r} gene fused to the *S. cerevisiae TEF2* (*ScTEF2*) promoter and terminator (*GEN3*) (Wendland et al., 2000); (3) the *Streptomyces noursei nat*^{r} gene flanked by the *S. cerevisiae PDC1* (*ScPDC1*) promoter and terminator (*NATPS*) (Hoepfner *in* Kaufmann, 2009); and (4) a *hyg*^{r} gene fused to the promoter and terminator sequences of the *A. gossypii LEU2* (*AgLEU2*) gene (Revuelta *in* Schlüpen, 2003; Jiménez et al., 2008). Because this last marker cassette contains sizeable homology regions to the *AgLEU2* locus, when used for targeted engineering it can direct integration into the *AgLEU2* locus rather than into the desirable target site.

The ability to rescue drug marker genes from the *A. gossypii* genome would therefore offer new perspectives in what concerns *A. gossypii* manipulation. Hence, the aim of this work was to develop a Cre-*lox*P-based system to remove and reuse selection markers in *A. gossypii*. To achieve this goal, several marker cassettes and Cre-expressing plasmids were constructed and successfully used to excise the selection markers used to delete two model genes, the *AgURA3* (*AEL059W*) gene and the predicted *AgADE1* (*AER221W*) open reading frame (ORF). These genes were chosen because their deletion not only should provide easy phenotype identification as would result in new auxotrophic strains, further expanding the molecular toolbox available for *A. gossypii*.

2. Materials and methods

2.1. Strains and media

A. gossypii ATCC 10895 strain, kindly provided by Prof. P. Philippsen (University of Basel) was used as the background for all deletions. Strains generated in this study are listed in Table 1. *A. gossypii* strains were grown at 30 °C on agar-solidified *Ashbya* full medium (AFM; 10 g/l tryptone, 10 g/l yeast extract, 20 g/l glucose, 1 g/l myo-inositol), containing 150 μg/ml geneticin (G418) (Sigma–Aldrich Química, Sintra, Portugal), 50 μg/ml nourseothricin (clonNAT) (WERNER BioAgents, Jena, Germany) or 5 μg/ml phleomycin (InvivoGen, Toulouse, France) for selection and maintenance of transformants. *A. gossypii* spores were prepared and stored as described by Ribeiro et al. (2010), with the exception that the mycelium was digested using 4 mg/ml Lysing Enzymes from *Trichoderma harzianum* (Sigma–Aldrich Química, Sintra, Portugal). Agar-solidified Synthetic Complete (SC) medium (Sherman et al., 1986) buffered with 0.1 M sodium-phosphate buffer (pH 7.0), lacking uracil and/or adenine, or supplemented with 5 mM uridine, uracil and adenine (AppliChem GmbH, Darmstadt, Germany) was used for phenotype characterization. When indicated, 5-fluoroorotic acid (5-FOA) (Sigma–Aldrich Química, Sintra, Portugal) was added to agar-solidified AFM at a final concentration of 1 mg/ml.

E. coli TOP10 (Invitrogen, Carlsbad, CA) served as plasmid host and was grown on agar-solidified LB medium (Sambrook and Russell, 2001) containing 100 μg/ml ampicillin (Sigma–Aldrich Química, Sintra, Portugal). Plasmids were maintained in a permanent culture at −80 °C in 2 ml aliquots with 15% (w/w) glycerol.

2.2. Molecular methods

DNA manipulations were carried out using standard procedures (Sambrook and Russell, 2001). Plasmid DNA was isolated from *E. coli* using the GenElute Plasmid Miniprep Kit (Sigma–Aldrich Química, Sintra, Portugal). PCR reactions were performed using NZYTaq DNA polymerase (NZYTech, Lisbon, Portugal) or Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland). All primers used in this work are listed in Table 2 and were obtained from Frilabo (Maia, Portugal). PCR fragments were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and DNA fragments were recovered from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The restriction enzymes used in this study were purchased from New England BioLabs (Ipswich, USA) or NZYTech (Lisbon, Portugal). T4 DNA ligase was purchased from Promega (Madison, USA).

Transformation of *A. gossypii* was carried out as described by Wendland et al. (2000), using 30 μg of DNA for integration of deletion cassettes, or 5 μg of DNA for plasmid insertion. *A. gossypii* genomic DNA was extracted as follows. A piece of mycelium collected from the border of *A. gossypii* colonies was transferred to microcentrifuge tubes containing 200 μl of autoclaved DNA extraction buffer (50 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris–HCl pH 8.0, and 0.5% (v/v) Triton X-100) and quickly mixed by vortexing. 100 μl of phenol:chloroform:isoamyl alcohol (25:24:1) were sub-

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant feature(s)	Reference
A. gossypii strains		
ATCC 10895	Parent strain	Prof. P. Philippsen
Agura3GEN3	Derived from ATCC 10895; *Agura3*Δ::*lox*P-*GEN3*-*lox*P	This work
Agade1NATPS	Derived from ATCC 10895; *Agade1*Δ::*lox*P-*NATPS*-*lox*P	This work
Agura3	Derived from *Agura3GEN3*; *Agura3*Δ::*lox*P	This work
Agade1	Derived from *Agade1NATPS*; *Agade1*Δ::*lox*P	This work
Agura3ade1GEN3	Derived from *Agura3*; *Agura3*Δ::*lox*P *Agade1*Δ::*lox*P-*GEN3*-*lox*P	This work
Agura3ade1	Derived from *Agura3ade1GEN3*; *Agura3*Δ::*lox*P *Agade1*Δ::*lox*P	This work
Plasmids		
pGEN3	*GEN3* cassette conferring resistance to G418: comprises the *E. coli kan*^{r} gene from transposon Tn*903* under the control of the *S. cerevisiae TEF2* promoter and terminator; ampicilin resistance	Wendland et al. (2000)
pUG66	*bleMX* cassette conferring resistance to phleomycin: comprises the bacterial *ble*^{r} gene from transposon Tn*5* under the control of the *A. gossypii TEF* promoter and terminator flanked by two *lox*P sequences; ampicillin resistance	Gueldener et al. (2002)
pUC19NATPS	*NATPS* cassette conferring resistance to clonNAT: comprises the *Streptomyces noursei nat1* gene flanked by the *S. cerevisiae PDC1* promoter and terminator; ampicillin resistance	D. Hoepfner *in* Kaufmann (2009)
pUGGEN3	Derivative of pUG66 with the *GEN3* cassette from pGEN3 instead of the *bleMX* cassette	This work
pUGNATPS	Derivative of pUG66 with the *NATPS* cassette from pUC19NATPS instead of the *bleMX* cassette	This work
pUGBLE3	Derivative of pUGGEN3 with the *ble*^{r} gene from pUG66 under the control of the *S. cerevisiae TEF2* promoter and terminator	Ribeiro et al. (2013)
pNatCre	*natMX* marker conferring resistance to clonNAT; comprises the *nat1* gene under the control of the *A. gossypii TEF* promoter and terminator; bacteriophage P1 *cre* gene under the control of the *S. cerevisiae GAL1* promoter and CYC1 terminator; *ARSH-CEN6* replication sequences from *S. cerevisiae*; ampicillin resistance	Steensma and Ter Linde (2001)
pAgNatCre	Derivative of pNatCre with the *cre* gene under the control of the *S. cerevisiae ADH2* promoter	This work
pAgBleCre	Derivative of pAgNatCre with the *bleMX* marker from pUG66 in place of the *natMX* cassette	This work

Table 2
Oligonucleotide primers used in this study. Underlined letters are nucleotide sequences complementary to the gene deletion regions. Recognition sequences for restriction enzymes are in bold.

Name	Sequence (5′–3′)
G1	GCTAGGGATAACAGGGTAAT
G2	CCG**CTCGAG**GATCTGATGAGGCCGTCT
G3	GATGGTGTAGGCTCCACATCACAGTAAGCATTTGTATAAGGCTGATCACATAGGGTGCTACCGACCCTAGCCATTGCCACGCAGGTCGACAACCCTTAAT
G4	GGGCATATAATTACAAAAAGGTTGCTGAGATGGTAATCGGCGGTGGAATCCGCCTATATACTGTGGGCGCCAGCGATAGAGCATAGGCCACTAGTGGATC
U1	CCATTGCCACATGTCAACGAAATC
U2	GTGTTGGATGCATGCTACTGAAC
G5	TCGCAGACCGATACCAGGATC
U3	AATCGCTCGAGCAACTCATTGG
N1	GA**AGATCT**TCCCTGCAGAACCGTTACGGTA
N2	CCG**CTCGAG**CCTGCAGCCAAACAGTGTT
N3	CCAGGCAGCGCGAGCTTGACTCTGGTCTATGGCCAATTTAACAGCCCCATATAGACAAGATATATATAGAGAGATAGCAGGCAGGTCGACAACCCTTAAT
N4	CACATCTCCATTTATCACTTAAAATTATTTAACATTTTAAAAGTACATCATTTTACCATTTCGCGCCTGTAAGTGCTTCGGCATAGGCCACTAGTGGATC
A1	CAATGCGTCTCGAGGAATTTGCTCG
A2	TCGGGTGACGTTAGCGCATCA
A3	AGAAGCAAGCGAGCGACTACG
N5	GATTCGTCGTCCGATTCGTC
AH1	C**GAGCTC**GCAAAGGGGCAAAACGTAG
AH2	CG**GAATTC**CGTGAGTTTCTGGAATAGACATTG
ScTEFt_EcoRI	CG**GAATT**CTCGATGAGTTTTTCTAAGAG
ScTEFp_NcoI	TCTATT**CCATG**GTAATTATAGTTCGTTGACCG
Ble	GCAGGTCGACAACCCTTAAT
Ble_EcoRI	CG**GAATTC**TCATGAGATGCCTGCAAG
qPCR*AgACT1*_F	TCGTTGCCACACGCCATTT
qPCR*AgACT1*_R	AAAGGAGTAGCCACGTTCCGATAG
qPCR*CRE*_F	CGTACTGACGGTGGGAGAATGTTA
qPCR*CRE*_R	TCCATCGCTCGACCAGTTTAGT

sequently added, followed by four vortexing cycles of 10 s each, with intercalary cooling on ice. After centrifugation for 15 min at maximum speed, 20 µl of the upper aqueous phase were diluted with 80 µl of water. 1–2 µl of these DNA samples were used as template for PCR amplification.

2.3. DNA constructs

The deletion cassettes *lox*P-*GEN3*-*lox*P and *lox*P-*NATPS*-*lox*P were constructed as follows. The *GEN3* cassette was amplified from p*GEN3* (Wendland et al., 2000) using primers G1 and G2 (Table 2) and the *NATPS* cassette amplified from pUC19NATPS (Hoepfner *in* Kaufmann, 2009) with primers N1 and N2 (Table 2). Both fragments were digested with *Bgl*II and *Xho*I, and cloned between the *lox*P sequences of *Bgl*II/*Xho*I digested pUG66 (Gueldener et al., 2002) to create pUGGEN3 and pUGNATPS, respectively. A *lox*P-*BLE3*-*lox*P cassette was also generated as described in Ribeiro et al. (2013). Briefly, the entire pUGGEN3 sequence, with the exception of the *kan*^{r} gene, was amplified with the primers ScTEFt_EcoRI and ScTEFp_NcoI (Table 2) and the *ble*^{r} gene obtained by PCR from plasmid pUG66 with the primers Ble and Ble_EcoRI (Table 2). Both PCR fragments were digested with *Eco*RI and *Nco*I and ligated to create pUGBLE3.

For deletion of the *AgURA3* (*AEL059W*) gene, the *lox*P-*GEN3*-*lox*P cassette was obtained by PCR from pUGGEN3 with primers G3 and G4 (Table 2). For deletion of the *AgADE1* (*AER221W*) ORF, either *lox*P-*GEN3*-*lox*P or *lox*P-*NATPS*-*lox*P cassettes were obtained by PCR from pUGGEN3 or pUGNATPS, respectively, with primers N3 and N4 (Table 2). The amplified deletion modules comprised the resistance cassettes flanked by *lox*P sequences and 80 bp guide sequences with homology to the 5′ and 3′ untranslated regions (UTRs) of the target loci, to ensure recombination in the correct location and direction in the genome.

Plasmids pAgNatCre and pAgBleCre were constructed from pNatCre (Steensma and Ter Linde, 2001), a shuttle vector that harbors *ARSH*-*CEN6* elements from *S. cerevisiae*, the *natMX* selective marker and the bacteriophage P1 *cre* gene flanked by the *S. cerevisiae GAL1* (*ScGAL1*) promoter and *CYC1* (*ScCYC1*) terminator. For that, a 599 bp fragment containing the *S. cerevisiae ADH2* (*ScADH2*) promoter, from position 872,712–873,310 on *S. cerevisiae* chromosome XIII, was amplified from the strain CEN.PK 113-7D genomic DNA with primers AH1 and AH2 (Table 2). The amplified fragment was digested with the restriction enzymes *Sac*I and *Eco*RI and cloned between the *Sac*I/*Eco*RI sites of pNatCre in place of the *ScGAL1* promoter. This new plasmid was named pAgNatCre. The pAgBleCre plasmid was obtained by the substitution of the *natMX* cassette from pAgNAtCre with the *bleMX* cassette from pUG66 with the restriction enzymes *Bgl*II and *Sac*I. All plasmids used and generated in this study are listed in Table 1.

2.4. Isolation of homokaryotic mutants and diagnostic PCR

Clonal selection of *A. gossypii* homokaryotic mutants was performed through the isolation of single uninucleated haploid spores from primary heterokaryotic transformants. For that, different dilutions of spores harvested from primary transformants were spread onto selective AFM plates with a guide line drawn in the bottom. After germination for approximately 24 h, plates were visually analyzed with a Nikon Diaphot 300 inverted microscope (Sony, Portugal) and single germlings were marked, isolated with a sterile scalpel and transferred to new selective AFM plates. The absence of the gene of interest was tested via diagnostic PCR.

PCR reactions were carried out in 25 µl reaction mixtures with 1–2 µl DNA template, 0.35 µM of each primer, 200 µM dNTP mix, 0.05 U/µl NZYTaq and NZYTaq buffer with 1.5 mM $MgCl_2$ provided by the manufacturer (NZYTech, Lisbon, Portugal). PCR conditions were: 5 min at 95 °C, 35 cycles of 95 °C for 45 s, 50 °C for 2 min (with increments of 0.3 °C per cycle), 72 °C for 1 min 30 s, and a final extension at 72 °C for 5 min. Correct integration of the *lox*P-*GEN3*-*lox*P cassette in the *AgURA3* and *AgADE1* loci was verified using the primer pairs U1/U2, G5/U2 and U3/U2 (Table 2), and A1/A2, G5/A3 and A1/A3 (Table 2), respectively. Integration of the *lox*P-*NATPS*-*lox*P cassette in the *AgADE1* locus was confirmed using the primer pairs A1/A2, A1/N5 and A1/A3 (Table 2).

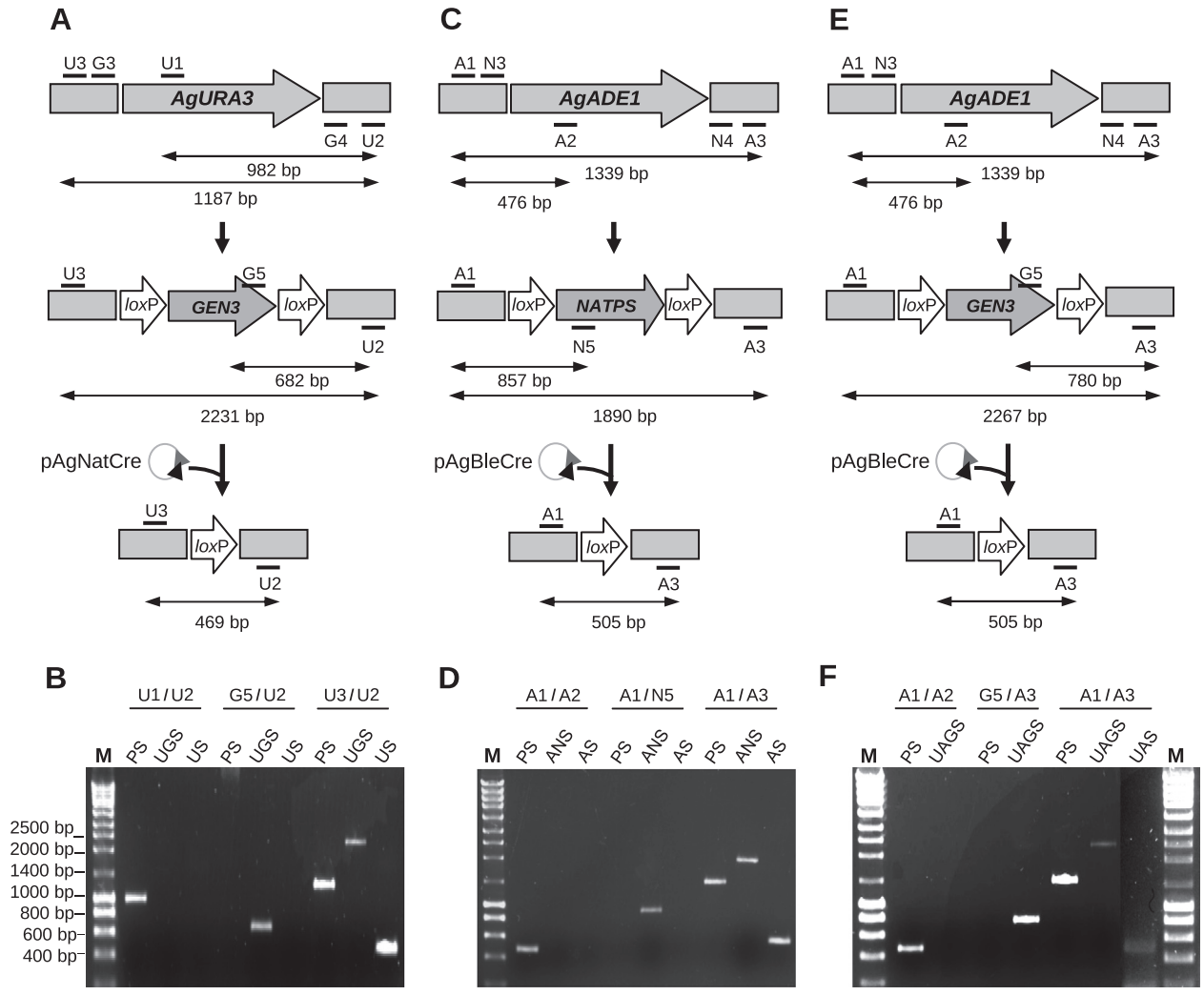


Fig. 1. Schematic representation of the strategy used for gene deletion and marker rescue in *A. gossypii*, and verification PCRs of the generated mutants, for which the *A. gossypii* ATCC 10895 parent strain (PS) was used as control. (A) Construction of the *A. gossypii Agura3* (US) marker-free strain. The *AgURA3* gene (*AEL059W*) was replaced by the *lox*P-*GEN3*-*lox*P cassette, which was amplified with the primer pair G3/G4, yielding the strain *Agura3GEN3* (UGS). This strain was subsequently transformed with the Cre-expressing plasmid pAgNatCre and excision of the *GEN3* marker occurred after transient expression of the Cre recombinase. (B) Verification of the integration of the *lox*P-*GEN3*-*lox*P cassette in the *AgURA3* locus and confirmation of its excision. (C) Construction of the *A. gossypii Agade1* (AS) marker-free strain. The *AgADE1* gene (*AER221W*) was replaced by the *lox*P-*NATPS*-*lox*P cassette, which was amplified with the primer pair N3/N4, yielding the strain *Agade1NATPS* (ANS)**.** This strain was transformed with the Cre-expressing plasmid pAgBleCre and the *NATPS* marker was excised as described above. (D) Verification of the integration of the *lox*P-*NATPS*-*lox*P cassette in the *AgADE1* locus and confirmation of its excision. (E) Construction of the marker-free *A. gossypii Agura3ade1* (UAS) double deletion strain. The *GEN3* marker, which had been excised from the *Agura3* strain, was used again to successfully delete the *AgADE1* gene in this strain, yielding the strain *Agura3ade1GEN3* (UAGS). This strain was transformed with the Cre-expressing plasmid pAgBleCre and the *GEN3* marker was again excised after transient expression of the Cre recombinase. (F) Verification of the integration of the *lox*P-*GEN3*-*lox*P cassette in the *AgADE1* locus of the *Agura3* strain and confirmation of its excision.

The positions of the primers and expected PCR amplicon sizes are given in Fig. 1.

2.5. Cre recombinase expression and marker cassette excision

For excision of the *lox*P-flanked marker cassettes, the mutant *Agura3GEN3* was transformed with the Cre-expressing plasmid pAgNatCre and the *Agade1NATPS* and *Agura3ade1GEN3* mutants with the plasmid pAgBleCre. The transformants were selected on agar-solidified AFM containing 50 g/l glucose and 50 μg/ml of clonNAT or 5 μg/ml of phleomycin, depending on the resistance conferred by the plasmid. To express the Cre recombinase, positive transformant colonies were grown for 1 week on agar-solidified AFM containing 10 g/l ethanol as sole carbon source plus the corresponding antibiotic selection. Simultaneously, the same transformants were also grown on selective AFM containing 50 g/l glucose, to test whether the expression of the Cre recombinase would be different in these conditions. Subsequently, spores harvested from these plates were spread onto agar-solidified AFM without selection, for plasmid loss. Small patches from the edge of each new mycelial colony were transferred to agar-solidified AFM containing G418 and/or clonNAT (depending on the resistance conferred by the deletion cassette used) and agar-solidified AFM without antibiotic. No growth in plates containing antibiotic indicated successful excision of the marker cassettes through recombination of the *lox*P sequences. The removal of the *lox*P-flanked marker cassettes was further confirmed by PCR and stable homokaryotic mutants free of exogenous markers were isolated as described above.

2.5.1. Quantitative PCR

For the expression analysis of the *cre* recombinase, mycelia from a colony of *Agura3GEN3* transformed with the Cre-expressing plasmid pAgNatCre were harvested (in triplicate) after 4 days of

growth on agar-solidified AFM containing 50 μg/ml clonNAT and either 10 g/l ethanol or 50 g/l glucose. Mycelia from untransformed *Agura3GEN3* grown for 4 days on agar-solidified AFM containing 10 g/l ethanol or 50 g/l glucose were also harvested in triplicate. Total RNA was extracted with the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) using the manufacturer's instructions for filamentous fungi and its concentration and purity was determined as previously described (Aguiar et al., 2014). After treatment with DNase I (Thermo Fisher Scientific, Loures, Portugal), 1 μg of total RNA was reverse transcribed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative PCR (qPCR) analyses were performed on a Bio-Rad (Hercules, CA) CFX 96 real-time PCR system as previously described (Aguiar et al., 2014), with the primer pairs shown in Table 2. Each cDNA (1:10) sample was analyzed in duplicate and the average cycle threshold was calculated. Relative expression levels were determined with the $2^{-\Delta\Delta CT}$ (Livak) method, using the experimentally determined amplification efficiency of the primers. The *cre* expression levels were normalized to the expression level of actin (*AgACT1*) for each strain in each condition.

3. Results

3.1. Constructs generated for marker recycling in A. gossypii

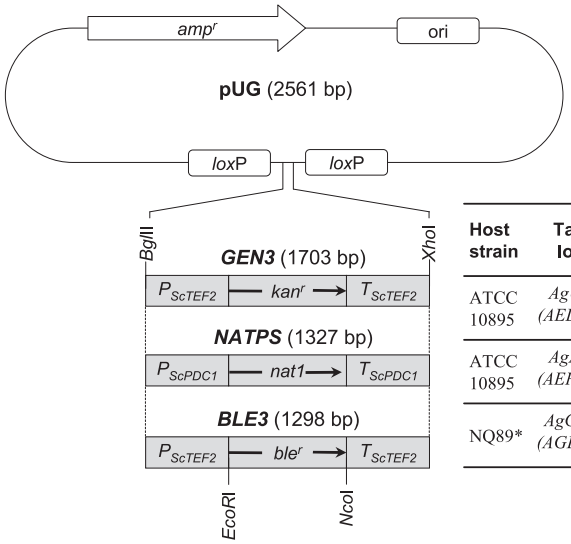
New disruption cassettes for use in *A. gossypii*, which could be excised after being used to screen for a genetic modification and then reused to produce multiple gene deletions in the same strain, were constructed using as backbone the plasmid pUG66 (Gueldener et al., 2002). The *lox*P-*GEN3*-*lox*P and *lox*P-*NAPS*-*lox*P cassettes were obtained by replacing the *bleMX* unit located between the two *lox*P sites in plasmid pUG66 with the *GEN3* and *NATPS* units from plasmids pGEN3 (Wendland et al., 2000) and pUC19NATPS (Hoepfner *in* Kaufmann, 2009), respectively, yielding plasmids pUGGEN3 and pUGNATPS (Fig. 2). The *ble*^{r} gene from transposon Tn*5*, which confers resistance to phlemoycin (Gatignol et al., 1987), was used to create the new heterologous dominant drug resistance marker cassette *lox*P-*BLE3*-*lox*P, as described in Ribeiro et al. (2013). The *ble*^{r} gene was isolated by PCR from plasmid pUG66 and placed between the *ScTEF2* promoter and terminator in plasmid pUGGEN3, yielding plasmid pUGBLE3 (Fig. 2).

New plasmids for transient expression of the Cre recombinase in *A. gossypii* were constructed using as backbone the replicative *ARSH-CEN6* plasmid pNatCre (Steensma and Ter Linde, 2001). This plasmid harbors a Cre expression cassette where the *cre* gene is flanked by the *ScGAL1* promoter and *ScCYC1* terminator sequences. Several *GAL* pathway genes are absent in the *A. gossypii* genome (Hittinger et al., 2004), including the *ScGAL1* homolog, hampering its growth on galactose. Hence, the *ScADH2* promoter was used instead to mediate the Cre recombinase expression in *A. gossypii*. In the plasmid pAgNatCre, the 467 bp *ScGAL1* promoter was therefore substituted with the 599 bp *ScADH2* promoter. Plasmid pAgBleCre was obtained by substituting the *natMX* drug marker in pAgNatCre with the *bleMX* expression cassette from plasmid pUG66. The usable range of phleomycin concentrations for selection of *A. gossypii* phleomycin-resistant colonies was determined and the optimal concentration for selection was found to be 5 μg/ml phleomycin (in agar-solidified AFM).

3.2. Deletion of the AgURA3 gene and generation of an uracil auxotrophic strain

As a model to test the functionality of the Cre-*lox*P system in *A. gossypii*, the entire coding region of the *AgURA3* (*AEL059W*) gene was deleted from the *A. gossypii* genome using the *lox*P-*GEN3*-*lox*P cassette flanked by 80 bp guide sequences with homology to the *AgURA3* 5′ and 3′ UTRs. Homologous integration of this deletion module within the *AgURA3* locus resulted in the replacement of *AgURA3* by the *lox*P-*GEN3*-*lox*P cassette with its transcriptional orientation corresponding to that of the deleted gene (Fig. 1A). Of the primary transformants randomly screened 53% were heterokaryotic, containing nuclei with both wild type and null alleles of *AgURA3* (Fig. 2). After a single spore purification step, diagnostic PCR indicated the sole presence of the mutant allele in the *Agura3GEN3* strain (Fig. 1B).

Deletion of the *AgURA3* gene caused uridine/uracil auxotrophy, being the homokaryotic *Agura3GEN3* strain unable to grow on



Host strain	Target locus	Primary Transformants			Efficiency (%)
		Obtained	Genotyped	Positive	
ATCC 10895	*AgURA3 (AEL059W)*	241	32	17	53
ATCC 10895	*AgADE1 (AER221W)*	292	36	2	6
NQ89*	*AgGAS1B (AGL352W)*	203	13	2	15

Fig. 2. Schematic illustration of the *lox*P-flanked marker modules constructed and corresponding targeting efficiencies, estimated by the ratio of the number of positive homologous integrants to the number of genotyped transformants. The marker cassettes *GEN3* and *NATPS* were PCR-amplified from pGEN3 and pUC19NATPS, and cloned between the *lox*P sites of pUG66 using the indicated enzymes. The *ble*^{r} gene was PCR-amplified from pUG66 and cloned between the *S. cerevisiae TEF2* promoter and terminator sequences of pUGGEN3. Three pUG plasmids containing the *GEN3* (pUGGEN3, 4264 bp), *NATPS* (pUGNATPS, 3888 bp) and *BLE3* (pUGBLE3, 3859 bp) cassettes flanked by *lox*P-sequences were obtained. *Ribeiro et al. (2013).

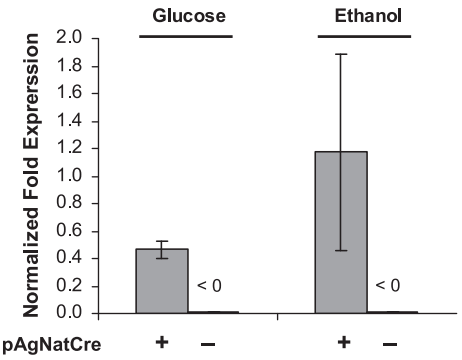


Fig. 3. qPCR analysis of the *cre* expression in *Agura3GEN3* transformed (+) or not (−) with the Cre-expressing plasmid pAgNatCre. The strains were grown for 4 days on agar-solidified AFM containing 50 g/l glucose or 10 g/l ethanol. The *cre* expression levels were normalized to the expression level of actin (*AgACT1*). Data represents the mean ± SD of three biological replicates.

defined medium lacking uridine and uracil (Fig. 4). When the medium was supplemented with uridine and uracil the *Agura3GEN3* strain grew similarly to the parent strain, which did not happen when the medium was supplemented with uracil alone (Fig. 4). Moreover, contrary to the parent strain, the *Agura3GEN3* strain was able to grow on agar-solidified AFM containing 1 mg/ml 5-FOA (Fig. 4).

3.3. Transient expression of Cre recombinase and marker rescue

The *Agura3GEN3* strain was transformed with the plasmid pAgNatCre to allow the transient expression of Cre recombinase. Transformants were selected on rich AFM containing clonNAT and 50 g/l glucose. Spores harvested from a randomly selected transformant colony were grown for 1 week on agar-solidified AFM containing clonNAT and 10 g/l ethanol as sole carbon source. The spores collected after this period were spread onto non-selective AFM, to promote plasmid loss, and the sensitivity of the resulting colonies to clonNAT and G418 was tested. Of the 39 mycelial colonies screened none was able to grow on AFM containing clonNAT, indicating that all had lost the plasmid that contained the *natMX* resistance marker after only one passage in non-selective conditions. Thirty-six colonies were also unable to grow on AFM containing G418, from which the *GEN3* marker had been looped out and lost. After a single spore purification step, the excision of the *GEN3* cassette from the *Agura3* locus was confirmed by PCR (Fig. 1B). This homokaryotic strain free of exogenous marker genes was named *Agura3*.

To test whether the excision of the *GEN3* cassette also occurred in glucose-containing medium, spores from the same randomly selected transformant colony were simultaneously grown on agar-solidified AFM containing clonNAT and 50 g/l glucose. In this case, 42 of the 49 mycelial colonies screened also lost their resistance to G418. Moreover, no statistically significant differences were found in the *cre* transcript levels between these conditions ($p = 0.159$), although a tendency was observed for lower expression of the Cre recombinase in glucose-containing medium (Fig. 3).

To assess the occurrence of background excision (i.e. Cre recombinase-independent excision), the untransformed *Agura3GEN3* strain was also grown for 1 week on agar-solidified AFM containing either 10 g/l ethanol or 50 g/l glucose. Of the 63 mycelial colonies screened for each condition none was sensitive to G418. Moreover, the transcript levels of the *cre* gene in this strain were undetectable (Fig. 3). Altogether, these data indicate that the excision of the *GEN3* cassette was dependent on the expression of the Cre recombinase.

3.4. Deletion of the AgADE1 gene and generation of a stable adenine auxotrophic strain

To demonstrate the versatility of the developed Cre-*lox*P system, the predicted *AgADE1* (*AER221W*) ORF was deleted using the *lox*P-*NATPS*-*lox*P cassette, with a targeting efficiency of 6% (Fig. 2). The correct integration of this deletion cassette was verified by PCR, as shown in Fig. 1D, and the resulting homokaryotic strain was named *Agade1NATPS*.

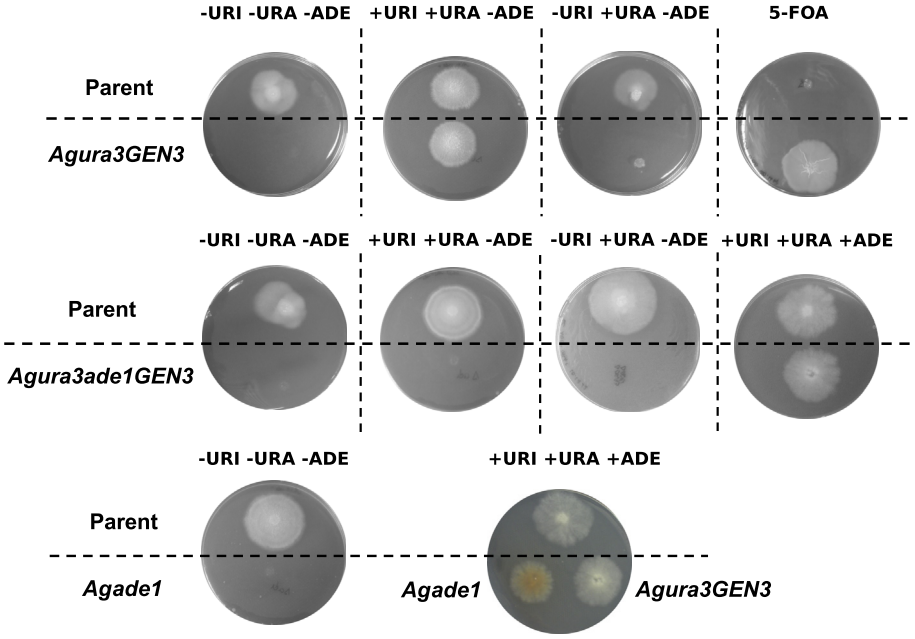


Fig. 4. Characterization of the *A. gossypii Agura3 and Agade1* null mutants growth on agar-solidified SC medium supplemented (+) or not (−) with uracil (URA), uridine (URI) and/or adenine (ADE). The *Agura3* mutants were also grown on agar-solidified AFM supplemented with 5-FOA (1 mg/ml). Plates were incubated for 3–5 days at 30 °C and the parental strain (ATCC 10895) was used as control. Red colored *Agade1* colonies were only observed when the concentration of adenine in the medium was lowered to 0.2 mM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

This strain was then transformed with the Cre-expressing plasmid pAgBleCre, transformants were selected on AFM containing phleomycin and 50 g/l glucose, and Cre recombinase was transiently expressed as described above. Following this strategy, the *lox*P-flanked *NATPS* cassette could be looped out of the chromosomal DNA of *Agade1NATPS* transformant colonies. After a single spore purification step, a homokaryotic *Agade1* strain free of marker genes (Fig. 1D) and auxotrophic for adenine (Fig. 4) was obtained.

3.5. Demonstration of marker recycling by deletion of the AgADE1 gene from the genome of the Agura3 strain

To demonstrate the successful recycling of the *GEN3* marker, the *AgADE1* ORF was also deleted from the genome of the *Agura3* strain using the *lox*P-*GEN3*-*lox*P cassette previously used to delete the *AgURA3* ORF, which was flanked by 80 bp guide sequences with homology to the *AgADE1* 5′ and 3′ UTRs. The correct integration of the deletion cassette in the *AgADE1* locus was verified by PCR as shown in Fig. 1F. Homokaryotic *Agura3ade1GEN3* mutants were obtained after a single spore purification step (Fig. 1F).

Physiological characterization of this mutant revealed that it was auxotrophic for both uridine/uracil and adenine (Fig. 4). This strain also continued to be resistant to 5-FOA (1 mg/ml).

To excise again the *GEN3* cassette from this strain, it was transformed with the Cre-expressing plasmid pAgBleCre for transient expression of the Cre recombinase, as described in Section 2. Of the 40 mycelial colonies screened after the transient expression protocol, 36 were unable to grow on AFM containing G418, suggesting that the *GEN3* marker had been lost again. After a single spore purification step, the excision of the *GEN3* cassette from the *Agade1* locus was confirmed by PCR (Fig. 1F). This homokaryotic strain free of exogenous marker genes was named *Agura3ade1*.

4. Discussion

Here, the Cre-*lox*P recombination system of the bacteriophage P1 was adapted to genetically engineer *A. gossypii* in a targeted way and allow the removal and reuse of the selection markers used during the process. Three heterologous recyclable drug marker cassettes and two Cre recombinase expression vectors for use in *A. gossypii* were created and successfully used to generate stable *Agura3* and *Agade1* auxotrophic strains free of exogenous selectable markers.

The *AgURA3* gene encodes the orotidine-5′-phosphate decarboxylase involved in the biosynthesis of pyrimidines (Pompejus et al., 1999) and its deletion caused uridine/uracil auxotrophy, and conferred resistance to 5-FOA. In the parent strain, growth was hampered by the presence of 5-FOA in the medium, which indicates that the AgUra3p converts 5-FOA into 5-fluorouracil, a toxic compound that causes cell death (Boeke et al., 1984). Therefore, the use of the *AgURA3* gene as a bidirectionally selectable marker (conferring uridine/uracil prototrophy and 5-FOA sensitivity) may be considered in future *A. gossypii* engineering projects. However, it is worth noting that in previous reports neither the *S. cerevisiae URA3* nor the *E. coli pyrF* genes were able to complement uracil auxotrophy in *A. gossypii Agura3* mutants (Pompejus et al., 1999). Therefore, efforts are now being made to develop an expression cassette that can complement the *AgURA3* auxotrophy.

Based on the analysis of the annotated genome sequence of *A. gossypii* (http://agd.vital-it.ch; Gattiker et al., 2007), the *AER221W* ORF (*AgADE1*) was found to be homologous to the *S. cerevisiae ADE1* gene. In *S. cerevisiae*, this gene encodes the *N*-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase involved in the purine biosynthetic pathway and its deletion causes adenine auxotrophy, and vacuolar accumulation of red-colored purine precursors when the exogenous adenine supplied is limited (Fischer, 1969). Both these phenotypes were also observed in the *A. gossypii Agade1* null mutants (Fig. 4). Previously, the deletion of the *AgADE4* gene had also been shown to cause adenine auxotrophy in *A. gossypii* (Jiménez et al., 2005), but not the red pigmentation phenotype characteristic of *ade1* and *ade2* mutants (Fischer, 1969).

For *S. cerevisiae*, several recyclable marker cassettes and Cre-expressing plasmids are available (Güldener et al., 1996; Steensma and Ter Linde, 2001; Gueldener et al., 2002). Unfortunately, these cannot be used in *A. gossypii* for several reasons. First, most of the available cassettes are not heterologous to the *A. gossypii* genome, as they contain the *A. gossypii TEF* promoter and terminator sequences. Second, the heterologous cassettes available are auxotrophic markers, therefore requiring the existence of auxotrophic strains. Third, the *cre* gene present in the available plasmids is under the regulation of the *ScGAL1* promoter (Steensma and Ter Linde, 2001; Gueldener et al., 2002), which cannot be used in *A. gossypii* because this fungus lacks pathways for the utilization of galactose in its genome (Dünkler and Wendland, 2007).

Therefore, the heterologous *GEN3* and *NATPS* markers widely used for PCR-based gene targeting in *A. gossypii* were adapted to contain *lox*P flanking sequences and successfully used to select *Agura3* and *Agade1* mutants. A new *lox*P-flanked heterologous drug resistance cassette was also constructed, which comprises the *ble*^{r} gene from transposon Tn*5* fused to the *ScTEF2* promoter and terminator (*BLE3*). This marker cassette renders resistance to phleomycin, a copper-containing antibiotic that acts by interfering with DNA synthesis (Gatignol et al., 1987). Although the *BLE3* marker was not used to delete any of the genes presented here, it was successfully used to generate and select *A. gossypii Aggas1agas1b* null mutants (Ribeiro et al., 2013), with a targeting efficiency of approximately 15% (Fig. 3).

In the generated Cre-expressing plasmids, the *ScGAL1* promoter was substituted by the *ScADH2* promoter to allow transient expression of the Cre recombinase in *A. gossypii*. In *S. cerevisiae*, the regulation of the *ScADH2* promoter is well studied, being its expression repressed several hundred-fold in the presence of glucose and activated when the glucose is depleted from the medium (Lee and DaSilva, 2005). In *A. gossypii*, as this promoter had never been used before little was known about its functionality, although previous observations had shown that the transcription of the *A. gossypii ScADH2* homolog (*AAR084W*) was also repressed by the presence of glucose in the medium (our microarray data). In this study, the expression of the Cre recombinase on agar-solidified AFM containing glucose was slightly lower than when ethanol was used instead as carbon source, but this difference was not statistically significant (Fig. 3). On the other hand, no *cre* transcripts were detected in strains lacking Cre-expressing plasmids, neither could be detected Cre recombinase-independent excisions of the marker cassettes. The constructed plasmids thus allowed the transient expression of the Cre recombinase, necessary to generate the mutant strains free of exogenous markers, which could be stopped after one subculturing step in unselective medium for plasmid loss.

In summary, the method here presented for multiple gene disruption and/or integration in *A. gossypii* offers advantages over the existing methods by enabling the creation of mutants entirely free of foreign genes and allowing the reuse of selection markers. The set of disruption cassettes and plasmids constructed greatly expand the possibilities for genetically engineer *A. gossypii*, being these suitable for use in both laboratorial and industrial strains, as they do not require any predetermined genetic background. Furthermore, since the number of stable *A. gossypii* auxotrophic strains currently available is limited, the strains generated here are envisaged to be useful for future genetic engineering projects. Efforts are now being made to develop a selection system based on the complementation of these auxotrophic mutants.

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