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RESEARCH

Molecular and Functional Characterization of an Invertase Secreted by *Ashbya gossypii*

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Abstract The repertoire of hydrolytic enzymes natively secreted by the filamentous fungus *Ashbya* (*Eremothecium*) *gossypii* has been poorly explored. Here, an invertase secreted by this flavinogenic fungus was for the first time molecularly and functionally characterized. Invertase activity was detected in *A. gossypii* culture supernatants and cell-associated fractions. Extracellular invertase migrated in a native polyacrylamide gel as diffuse protein bands, indicating the occurrence of at least two invertase isoforms. Hydrolytic activity toward sucrose was approximately 10 times higher than toward raffinose. Inulin and levan were not hydrolyzed. Production of invertase by *A. gossypii* was repressed by the presence of glucose in the culture medium. The *A. gossypii* invertase was demonstrated to be encoded by the *AFR529W* (*AgSUC2*) gene, which is highly homologous to the *Saccharomyces cerevisiae SUC2* (*ScSUC2*) gene. *Agsuc2* null mutants were unable to hydrolyze sucrose, proving that invertase is encoded by a single gene in *A. gossypii*. This mutation was functionally complemented by the *ScSUC2* and *AgSUC2* genes, when expressed from a 2-μm-plasmid. The signal sequences of both AgSuc2p and ScSuc2p were able to direct the secretion of invertase into the culture medium in *A. gossypii*.

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Introduction

The filamentous hemiascomycete *Ashbya gossypii* (syn. *Eremothecium gossypii*), a well known riboflavin overproducer [1], was sequenced in 2004 [2]. The remarkably high degree of gene homology and gene order conservation existent between its genome and the genome of the baker's yeast, *Saccharomyces cerevisiae* [2, 3], has facilitated the assignment of potential functions to *A. gossypii* open reading frames (ORFs). However, up to now only a small percentage of ORFs have been experimentally characterized in *A. gossypii*. Furthermore, although this fungus has been recently considered as a host for the expression and secretion of recombinant proteins [4], only one native protein secreted by *A. gossypii*, a lipase, has been characterized so far [5]. Nevertheless, extracellular amylase [6, 7] and β-glucosidase [7] enzymatic activities have been detected in *A. gossypii* ATCC 10895 culture supernatants. The proteins responsible for the detected activities have not yet been characterized.

Invertase, or β-fructofuranosidase, (EC 3.2.1.26) is an industrially important enzyme secreted by many fungi that has wide applications in the food, pharmaceutical, and bioethanol production sectors. It catalyzes the release of terminal β-fructose residues from various β-D-fructofuranoside substrates, such as sucrose and raffinose. Fungal invertases have been widely studied in yeast [8–11] and filamentous fungi [12–15], and based on the homology of their aminoacid sequences they have been classified within the family 32 of the glycoside hydrolases (GH32) [16].

Ashbya gossypii is able to utilize sucrose as carbon source [17, 18], which should reflect the presence of either intra- or extracellular invertase activity in this fungus. A putative invertase-enconding gene, homologous to the *S. cerevisiae SUC2* (*ScSUC2*) gene, can be identified in its genome. The deduced amino acid sequence of the protein it presumably encodes also shares identity with that of the *S. cerevisiae* Suc2 invertase (ScSuc2p).

The *ScSUC2* gene encodes two different invertase isoforms: a constitutively expressed cytoplasmic form, which is non-glycosylated, and a glucose-repressible glycosylated form, which is secreted into the periplasmic space [19, 20]. Several factors affect invertase secretion in different fungi, and the carbon source used has been found to play an important role as repressor or inducer of its synthesis [21, 22].

Here, we describe, for the first time, the molecular and functional characterization of an invertase secreted by *A. gossypii*. Studies of its secreted and cell-associated hydrolytic activities were performed, and the functionality of the *A. gossypii ScSUC2* homolog gene (*AgSUC2*) was demonstrated through its deletion and complementation by recombinant expression.

Materials and Methods

Strains

Ashbya gossypii ATCC 10895 was obtained from Prof. Peter Philipsen (University of Basel). *A. gossypii* strains *Agsuc2* (*Agsuc2::GEN3*), *Agsuc2*pTAgSUC (*Agsuc2::GEN3*, pTAgSUC) and *Agsuc2*pTScSUC2 (*Agsuc2::GEN3*, pTScSUC2) were generated in this study using the ATCC 10895 strain as the parent. *Escherichia coli* TOP10 (Invitrogen) was used as the recipient for all cloning steps.

Media and Culture Conditions

Escherichia coli was grown at 37 °C in LB medium supplemented with 100 μg/ml of ampicillin (Sigma-Aldrich) for selection. *A. gossypii* was maintained on agar (15 g/l agar) solidified *Ashbya* full medium (AFM; 10 g/l yeast extract, 10 g/l tryptone, 1 g/l myo-inositol, 20 g/l glucose). Spore suspensions were prepared and stored as described by Ribeiro et al. [4], except that the mycelium was digested using 4.5 mg/ml lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich). For selection of *A. gossypii* transformants, the antibiotics geneticin (G418) (Sigma-Aldrich) or nourseothricin (clonNAT) (WERNER BioAgents) were used at a final concentration of 200 and 100 μg/ml, respectively. In liquid medium, selection was maintained with 50 μg/ml of clonNAT.

Submerged cultures were inoculated with 10^6 spores and grown at 30 °C and 200 rpm in 250-ml Erlenmeyer flasks containing 50 ml of AFM or synthetic complete (SC) medium [23] with 20 g/l of glucose, sucrose or glycerol as carbon source. 0.1 M sodium-phosphate at pH 7.0 was used to buffer SC medium. For bioreactor cultivations, precultures were grown in liquid AFM for 17 h before the mycelia were harvested by filtration through disks of sterile disposable cleaning cloth (X-tra, Inex Partners Oy) and washed with sterile distilled water. Bioreactors (Sartorius AG, 2 l Biostat® B-DCU, 1.0 l working volume) containing defined minimal medium [24] with 20 g/l of sucrose as carbon source were inoculated to an initial biomass of 0.62 ± 0.05 g/l. Polypropylene glycol (mixed molecular weights [25]) was added to a final concentration of 0.2 % (v/v) to prevent foam production. Cultures were grown at 30 °C, with 500 rpm agitation and aeration of 1 volume of air per volume of liquid per minute (vvm). Culture pH was maintained at 6.0 ± 0.1 by the addition of sterile 1 M KOH or 1 M H_3PO_4.

Analyses

Culture optical density at 600 nm (OD_{600}) was used to monitor growth of submerged cultures. Biomass concentration was determined as dry cell weight. Mycelium was collected by filtration through disks of disposable cleaning cloth (X-tra, Inex Partners Oy) or filter paper (Advantec qualitative grade 2), washed with two sample volumes of distilled water and dried to a constant weight at 105 °C.

Supernatant was obtained by filtration of culture broth through 0.2 μm cellulose acetate filters (Whatman or Advantec) for analysis of enzymatic activities, substrates and metabolites (sucrose, glucose, fructose, glycerol and ethanol). Substrates and metabolites were quantified by high performance liquid chromatography (HPLC) using a Fast Acid Analysis Column (100 mm × 7.8 mm, Bio-Rad) linked to an Aminex HPX-87H Organic Acid Analysis Column (300 mm × 7.8 mm, Bio-Rad) or a MetaCarb 87H column (300 mm × 7.8 mm; Varian) at 35 °C, with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 ml/min.

To assess biomass associated invertase activity, biomass was collected by filtration, washed with cold 10 mM sodium azide in 0.05 M phosphate-citrate buffer (pH 6.0) and then resuspended in 0.5 ml of the same solution.

For gene expression analyses, mycelial samples were collected from flask cultivations in AFM containing glycerol as primary carbon source during exponential growth (24 and 25 h after inoculation). After 24 h, glucose was added to three out of six *A. gossypii* cultures at a final concentration of 20 g/l. To the other three cultivations, an equivalent volume of H_2O was added. Samples were

collected immediately before (culture time 24 h) and 1 h (culture time 25 h) after addition of glucose/H_2O. Mycelium was rapidly separated from the culture supernatant by filtration through filter paper, washed with two sample volumes of 0.9 % (w/v) NaCl and stored immediately at −80 °C.

Invertase Assays

To determine the secreted and extracellular cell wall-bound invertase activity, 50 µl of filtered supernatant or treated mycelial suspensions were mixed with 100 µl of 0.1 M phosphate-citrate buffer (pH 6.0) and 50 µl of 0.2 M sucrose. For total cell-associated invertase activity (including both intracellular and extracellular cell wall-bound invertase), 250 µl of resuspended mycelia were first disrupted with 0.25 g of 20 mm diameter glass beads (Sigma-Aldrich) using a FastPrep FP120 (Qbiogene; 4 cycles at speed 6 for 10 s, with ice cooling between cycles). Resuspended lysed mycelia (50 µl) were then used for the assay. The reactions were carried out at 40 °C for 90 min and stopped by boiling for 10 min. As a blank, each reaction mixture was incubated without the sample, which was only added to the mix immediately before stopping the reaction. Substrate specificity assays were performed with culture filtrates as described above, but using 50 µl of 0.2 M raffinose, 1 % (w/v) levan or 1 % (w/v) inulin as substrate instead of sucrose.

The amount of reducing sugars formed was determined by the 3,5-dinitrosalicylic acid (DNS) method [26], using glucose as standard. One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyzed sucrose to yield 1 µmol of glucose (or reducing sugars, for the other substrates) per minute under the stated conditions. Specific enzyme activities are expressed as U per gram of dried biomass (U/g).

Gene Expression Analyses

Total RNA was extracted from frozen mycelium using the RNeasy Plant Mini kit (QIAGEN) according to the manufacturer's instructions for filamentous fungi and treated with DNase I (Fermentas). The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000^{TM} (Thermo Scientific) and its integrity assessed on 1.5 % agarose gel by visualization of the 28S/18S rRNA banding pattern after eletrophoresis. 500 ng of total RNA were reverse transcribed (RT) using the NZY First-Strand cDNA Synthesis Kit (NZYTech). Quantitative PCR analyses were performed on a Bio-Rad CFX 96 real-time PCR system. Each sample was tested in duplicate in 10 µl reaction mixes containing 5 µl of SsoFast EvaGreen Supermix (Bio-Rad), 6 pmol of each qPCR primer (Table 1) and 1 µl of diluted (1/5) cDNA or no-RT control. The thermocycling program consisted of one hold at 95 °C for 30 s, followed by 39 cycles of 95 °C for 5 s and 64 °C for 5 s. Quantitative PCR products were analyzed by melting curves for unspecific products or primer dimer formation. Relative expression levels were determined with the $2^{-\Delta\Delta C}{}_T$ (Livak) method, using the experimentally determined amplification efficiency of the primers. For standardization, the results were expressed as ratios of target gene expression to the reference gene (*AgACT1*) expression.

Electrophoretic Analyses

The proteins secreted by *A. gossypii* to the cultures supernatants were concentrated using Amicon® Ultra-15 10,000 MWCO centrifugal filter devices (Millipore) according to the manufacturer's instructions. Soluble mycelial fractions for electrophoretic analyses were prepared as follows. The mycelium from 12 flask cultivations was harvested by filtration through 0.2 µm cellulose acetate filters (Advantec), digested with 4.5 mg/ml lysing enzymes from *T. harzianum* (Sigma-Aldrich) for 2 h and filtered again. The soluble filtrate obtained was concentrated to 1 ml using Amicon® Ultra-15 10,000 MWCO centrifugal filter devices (Millipore). For buffer exchange, the concentrated fractions were repeatedly diluted with 0.05 M phosphate-citrate buffer (pH 6.0) and concentrated again. All procedures were carried out at 4 °C.

Invertase activity was detected in situ after native polyacrylamide gel electrophoresis (PAGE) on 8 % (w/v) polyacrylamide gels. After incubation for 24 h at 30 °C in 0.1 M phosphate-citrate buffer (pH 6.0) containing 0.2 M sucrose, the reducing sugars in the gels were stained with 0.1 % (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) in 0.25 M NaOH. Sucrolytic activity was reflected by the formation of red formazan bands resulting from the reaction of TTC with the reducing sugars. The reaction was stopped with 7.5 % (v/v) acetic acid. Invertase from *S. cerevisiae* (Sigma-Aldrich # I4504) was used as a positive control.

Plasmid Constructions

Expression plasmids containing the *S. cerevisiae* 2-µm replication origin were generated as follows. The *NATPS* cassette conferring resistance to clonNAT was amplified from pUC19NATPS (Hoepfner in [27]) with the primers N1/N2 (Table 1). The *kan*MX and the *S. cerevisiae URA3* expression modules in pMI516 [4] were exchanged with the *NATPS* module using the *Bgl*II/*Bgl*II sites, generating plasmid pMINATPS. Based on the annotated sequence for the *AFR529W* (*AgSUC2*) OFR in the AGD database (http://

Table 1 Primers used in this study

Primer	Sequence (5′–3′)
N1—NATPS-BglII_FW	gaagatcttcCCTGCAGAACCGTTACGGTA
N2—NATPS-BglII_RV	gaagatcttcCCTGCAGCCAAACAGTGTT
I1—INV-EcoRI_FW	cggaattccgTGCATCACTTAACATCAATCAGCA
I2—INV-EcoRI_RV	cggaattccGCGCACGTATTGTGCTTTACTAG
S1—SUC2-EcoRI_FW	cggaattccgAAAGCTTTTCTTTTCACTAACG
S2—SUC2-EcoRI_RV	cggaattccgCCTTTAGAATGGCTTTTGAA
P1—PGK_FW	GTTTAGTAGAACCTCGTGAAAC
P2—PGK_RV	GGCATTAAAAGAGGAGCG
G1—GEN3_FW	**cgactgcgataagagatgcatcacttaacatcaatcagca**GCTAGGGATAACAGGGTAAT
G2—GEN3_ RV	**ggaacactctacgtaggcgcacgtattgtgctttactaga**AGGCATGCAAGCTTAGATCT
V1—INV_FW	GAGGCCGTTGTCGTGTAGAG
V2—Kan_RV	GTTTAGTCTGACCATCTCATCTG
V3—Kan_FW	TCGCAGACCGATACCAGGATC
V4—INV_RV	TCCGGAACATCACATAAGCA
V5—INV_FW	GTTCCAGGGATTCTACAACA
qPCR*AgACT1*_FW	ACGGTGTTACCCACGTTGTTCC
qPCR*AgACT1*_RV	TCATATCTCTGCCGGCCAAGTC
qPCR*AgSUC2*_FW	AACGACTCTGGCGCTTTCTCT
qPCR*AgSUC2*_RV	TCTCGGATTCAGGCGTGTTGT

Upper case sequences correspond to sequences complementary to the template. Lower case sequences correspond to additions for restriction sites (underlined) or to the disruption cassette sequences with homology to *AgSUC2* flanking regions (bold)

agd.vital-it.ch [28]) and *YIL162W* (*ScSUC2*) ORF in the SGD database (www.yeastgenome.org), the complete coding regions of *AgSUC2* (1719 bp) and *ScSUC2* (1599 bp) were amplified by PCR from *A. gossypii* ATCC 10895 and *S. cerevisiae* CEN.PK 113-7D genomic DNA using the primers I1/I2 and S1/S2 (Table 1), respectively. These fragments were cloned into the *Eco*RI site of pMINATPS, between the *ScPGK1* promoter and terminator sequences. The resulting plasmids were named pTAgSUC and pTScSUC2, respectively. The sequence and orientation of the inserts in the plasmids were confirmed by sequencing (Eurofins MWG Operon) with primers P1, P2, I1, and I2 (Table 1).

Ashbya gossypii Transformation

For the deletion of the complete coding region of the *AgSUC2* gene, a disruption cassette containing the *GEN3* expression module (conferring resistance to G418) flanked by 40 bp sequences with homology to the upstream and downstream regions of the *AgSUC2* ORF was obtained by PCR with primers G1/G2 (Table 1), using pGEN3 [29] as template. The amplified cassette was purified with the QIAquick PCR purification Kit (Qiagen) and used to transform *A. gossypii* by electroporation, as described in Wendland et al. [29]. Homokaryotic *Agsuc2* null mutants were obtained through the isolation of single spores from primary heterokaryotic transformants, which were germinated under selective conditions. Diagnostic PCR with primers V1/V2, V3/V4, and V5/V4 (Table 1) was used to verify the correct integration of the disruption cassette and absence of the target gene in homokaryotic deletion mutants.

Ashbya gossypii Agsuc2 null mutants were transformed by electroporation [29] with plasmids pTAgSUC, pTScSUC2 and pMINATPS (empty vector) and positive clones were selected in AFM containing clonNAT. Additionally, the ability of the clones to use sucrose as sole carbon source was assessed in flask cultivations with AFM containing either glucose or sucrose. Two strains, one expressing the homologous AgSuc2 invertase and other expressing the heterologous ScSuc2 invertase, were selected for further study.

Bioinformatic Analyses

The deduced amino acid sequence encoded by the putative invertase gene from *A. gossypii* (GenBank accession no. AAS53900.1) was analyzed by using the BLAST server against the *S. cerevisiae* S288c invertase (GenBank accession no. DAA08390.1), and the two sequences were aligned using the Clustal Omega [30] server (http://www.ebi.ac.uk/Tools/msa/clustalo/; default parameters).

Prediction of signal peptides was done using the SignalP 3.0 [31] server (www.cbs.dtu.dk/services/SignalP-3.0/) and prediction of putative *O*- and *N*-glycosylation sites was done using the NetOGlyc 3.1 [32] (www.cbs.dtu.dk/services/NetOGlyc/) and NetNGlyc 1.0 [33] (www.cbs.dtu.dk/services/NetNGlyc/) servers, respectively.

Results and Discussion

Invertase Production by *A. gossypii*

The filamentous fungus *A. gossypii* is able to consume sucrose as carbon source [17, 18], but invertase activity had not yet been described from this organism. We observed invertase activity in the culture supernatant, as well as associated with cell biomass (Fig. 1). Substrate specificity assays revealed that *A. gossypii* culture filtrates displayed approximately 10 times more hydrolytic activity toward sucrose (213 ± 4 mU/l) than toward raffinose (21 ± 1 mU/l), whereas inulin and levan were not hydrolyzed. Sucrose was subsequently used as substrate in all enzymatic assays.

In bioreactor cultivations of *A. gossypii* mycelium in defined minimal medium containing sucrose as carbon source, maximum specific invertase activities (36 ± 1 to 48 ± 1 U/g) were detected in the culture supernatant at the stationary phase (Fig. 1a), when sucrose, glucose, and fructose had been depleted from the medium (Fig. 1b). These levels of activity were about threefold higher than those quantified when sugars were still present in the medium and were maintained for about 25 h of culture before slowly decreasing. In a similar way, the invertase activity of the total cell-associated fraction (which included both intracellular and extracellular cell wall-bound invertase) also reached the maximum levels during this period (76 ± 6 to 91 ± 12 U/g), being approximately twofold higher than the activity detected in the culture supernatant (Fig. 1a). A prominent increase in the invertase activity from both culture supernatant and total cell-associated fraction was observed between 15 and 20 h of culture (Fig. 1a). This coincided with the depletion of the glucose and fructose from the medium (Fig. 1b). Similar observations were made from bioreactor cultivations in AFM with sucrose as the primary carbon source for cell-associated invertase activity. In the culture supernatant only low invertase activity (<5 U/g) was observed (data not shown).

The patterns of secreted and cell-associated invertase activity observed during *A. gossypii* bioreactor cultivations in both defined and complex medium suggested that the presence of glucose and/or fructose in the medium may repress invertase production in *A. gossypii*, as observed in *S. cerevisiae* [21], *Schizosaccharomyces pombe* [10], *Aspergillus nidulans* [12], and *A. niger* [22]. Glucose repression in fungi is mediated by the binding of regulatory proteins (Mig1p and Mig2p in *S. cerevisiae* [20, 21], CreA/Cre1 in *A. nidulans* and *Trichoderma reesei* [34–37]) to GC-rich consensus sequences ([G/C][C/T]GGGG in *S. cerevisiae*, [G/C][C/T]GG[A/G]G in *A. nidulans* and *T. reesei*) in the promoter region of the regulated gene. In *S. cerevisiae*, the presence of an AT-rich sequence 5′ to the recognition site of Mig1p is essential for its binding [38]. In

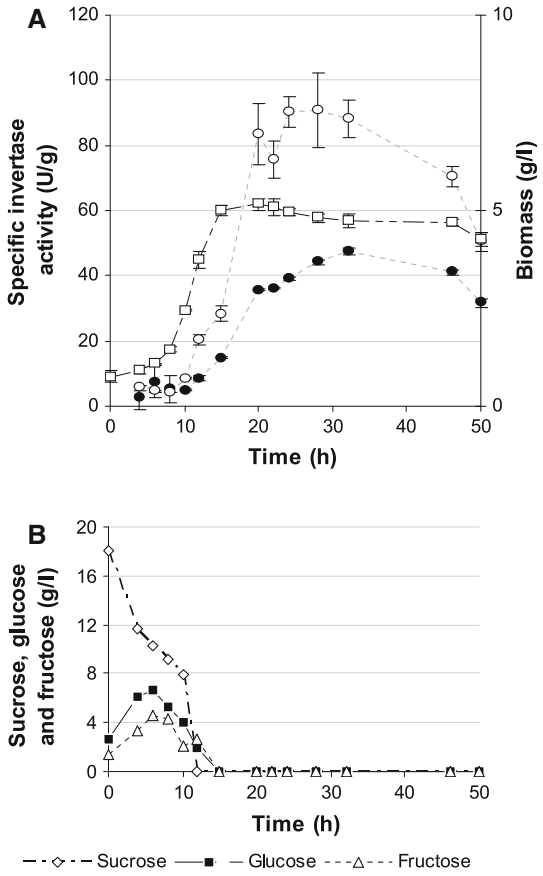

Fig. 1 Secreted (*filled circle*) and cell-associated (*open circle*) specific invertase activity during *A. gossypii* ATCC 10895 growth in defined minimal medium containing sucrose as primary carbon source, at pH 6.0, 30 °C, 500 rpm with 1 vvm aeration. **a** Specific invertase activity (*circles*) is read on the primary axis and biomass concentration (*squares*) on the secondary axis. *Error bars* represent the standard error of the mean of three independent measurements. **b** The sugar concentration in the culture supernatants at each time point the activities in **a** were measured

silico analysis of the deduced 5′ untranslated region (UTR) of the *A. gossypii* homolog to the *ScSUC2* gene (*AFR529W*) revealed the presence of four putative binding sites for a catabolite repressor ([G/C][C/T]GGGG) preceded by an AT-rich sequence (Fig. 2). These should be recognition sites for binding of the *A. gossypii* Mig1p homolog (AFR471C), as the zinc-finger domains of the AgMig1p and ScMig1p are highly similar. Interestingly, in bioreactor cultivations using AFM with glucose as primary carbon source, the level of the *AgSUC2* mRNA transcript increased 1.8-fold ($p < 0.002$) after glucose was depleted from the culture medium, when compared with the transcript levels measured when glucose was present at least from 17 ± 1 to 7 ± 2 g/l (our microarray data). Moreover, addition of 20 g/l of glucose to flask cultivations during exponential growth in AFM containing glycerol as primary carbon source resulted in a 4.8-fold decrease ($p < 0.038$) of

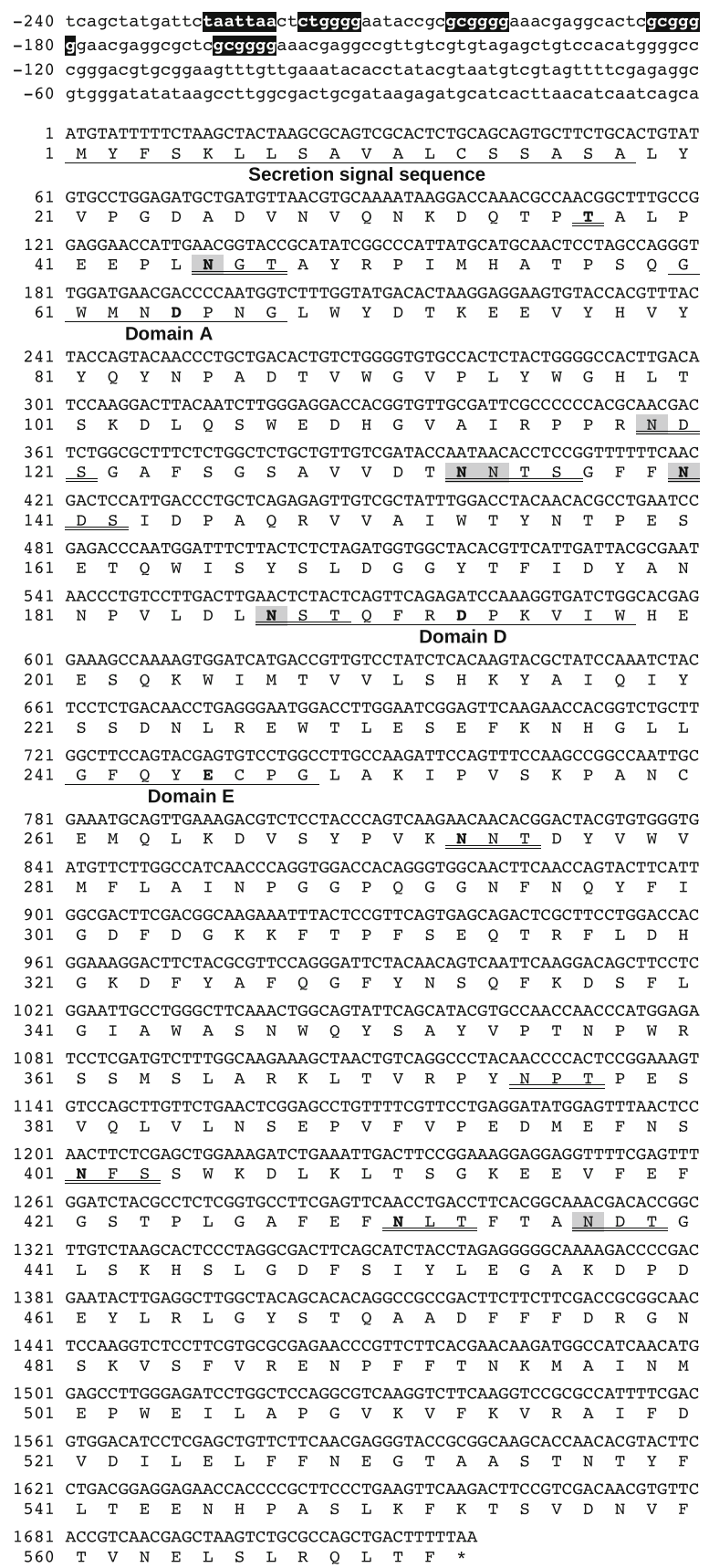

Fig. 2 Nucleotide and deduced amino acid sequence of the *AgSUC2*-encoded invertase and its 5′ untranslated region (UTR). Potential recognition sites for AgMig1p binding and the preceding A–T-rich sequence in the *AgSUC2* 5′ UTR are blocked in *black*. The putative secretion signal peptide and the catalytic domains A, D and E are *underlined*, with the amino acids of the catalytic site marked in *bold*. Potential *O*-glycosylation (S/T) and *N*-glycosylation sites (N-X-S/T, X any amino acid except proline) are marked by *two lines*. *N*-glycosylation sites conserved in ScSuc2p are highlighted in *gray* and sites with higher probability of being occupied represented in *bold*

Table 2 Relative expression of *AgSUC2* during exponential growth of *A. gossypii* ATCC 10895 in AFM containing glycerol as primary carbon source, immediately before (24 h of culture time) and 1 h (25 h of culture time) after addition of 20 g/l of glucose to the culture medium

	Culture time		
	24 h[a]	25 h[b]	
		Without glucose	With glucose
% of *AgSUC2* expression	100 ± 43	100 ± 30	21 ± 5

Expression levels of *AgSUC2* were normalized using *AgACT1* as the reference gene. Cells growing without addition of glucose were used as the calibrator sample (the expression was set at 100 %). Results shown are mean ± SD of results from six or three independent cultivations. Each sample (one sample from each biological replica) was analyzed in duplicate, and the coefficient of variation (after normalization) between the results for these technical duplicates was <30 %

[a] Six independent cultivations

[b] Three independent cultivations

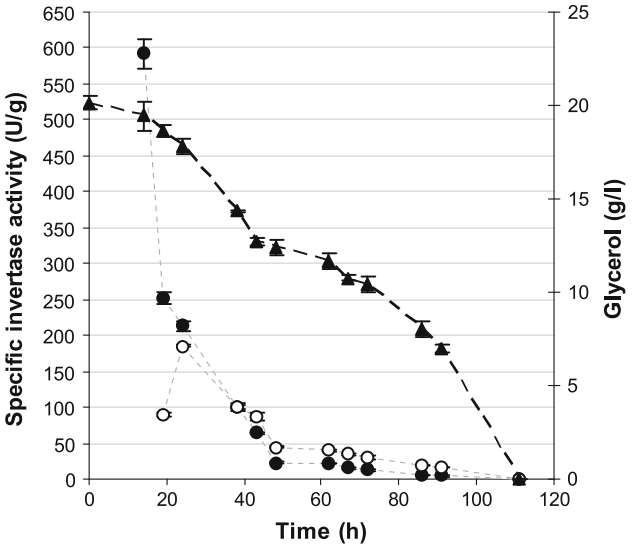

Fig. 3 Specific invertase activity in the culture supernatant (*filled circles*) and total cell-associated fraction (*open circles*) during growth of *A. gossypii* ATCC 10895 in 250-ml flasks containing 50 ml of AFM with glycerol as primary carbon source. Specific invertase activity is read on the primary axis and glycerol concentration on the secondary axis. *Error bars* represent the standard error of the mean of three independent biological replicates and where not seen were smaller than the symbol

the *AgSUC2* expression (Table 2). Altogether, these results indicate that glucose represses *AgSUC2* transcription and that sucrose is not needed as an inducer.

In flask cultivations of *A. gossypii* in AFM containing glucose as primary carbon source, invertase activity was found in the supernatant and cell-associated fractions during growth when glucose was present in concentrations as high as 15 g/l (data not shown). However, the maximum specific invertase activity in the supernatant of these cultures (19 ± 2 U/g) was approximately 30-fold lower than that quantified in glycerol cultures (592 ± 21 U/g) (Fig. 3). These observations reinforce the suggestion that the production of secreted invertase by *A. gossypii* is repressed by the presence of fermentable sugars in the medium and that sucrose is not needed as an inducer.

In contrast to the high specific invertase activity observed during the deceleration and stationary phase in bioreactor cultivations (Fig. 1), specific activity in flask cultivations with either fermentable (data not shown) or non-fermentable carbon sources (Fig. 3) was highest during the growth phase and decreased during stationary phase. Thus, the extent to which invertase should be considered to be growth associated in *A. gossypii* is still unclear.

Unexpectedly, when *A. gossypii* spores (derived from the same stocks) were grown in SC medium containing glucose as carbon source, the lag phase was much longer than when sucrose was used (Fig. 4). No difference in the lag phase for cultures growing on glucose or sucrose has been observed in AFM (data not shown). The long lag phase in SC may reflect differences in the osmolarity of SC medium and AFM, or increased sodium toxicity from the sodium phosphate buffer which was used to buffer SC medium but not AFM, since both high osmolarity and sodium toxicity have been observed to increase the lag phase of *A. gossypii* [39]. Regardless, sucrose was beneficial for the initial stages of *A. gossypii* growth in SC medium, possibly because it contributed less to the osmotic pressure of the medium than did the glucose.

Molecular and Functional Characterization of the *A. gossypii* Invertase

In silico analysis of the *A. gossypii AFR529W* ORF (*AgSUC2*) showed that its 1719 bp DNA sequence putatively encodes a protein with 572 amino acids and a calculated molecular mass of 64.99 kDa, which shares 53 % of amino acid identity with the ScSuc2p. Moreover, the conserved domains A, D, and E, which contain highly conserved acidic residues located in the active site of GH32 members [40], were all present in AgSuc2p (Fig. 2). Thus, we expected that this protein was responsible for the hydrolysis of sucrose to fructose and glucose in *A. gossypii*.

To investigate the function of the *AgSUC2* gene, its complete coding region was deleted from the *A. gossypii* genome through a one-step PCR-based gene targeting approach [29] and the *Agsuc2* null mutants were physiologically characterized (Fig. 5). These mutants lost their ability to grow on sucrose as sole carbon source, but

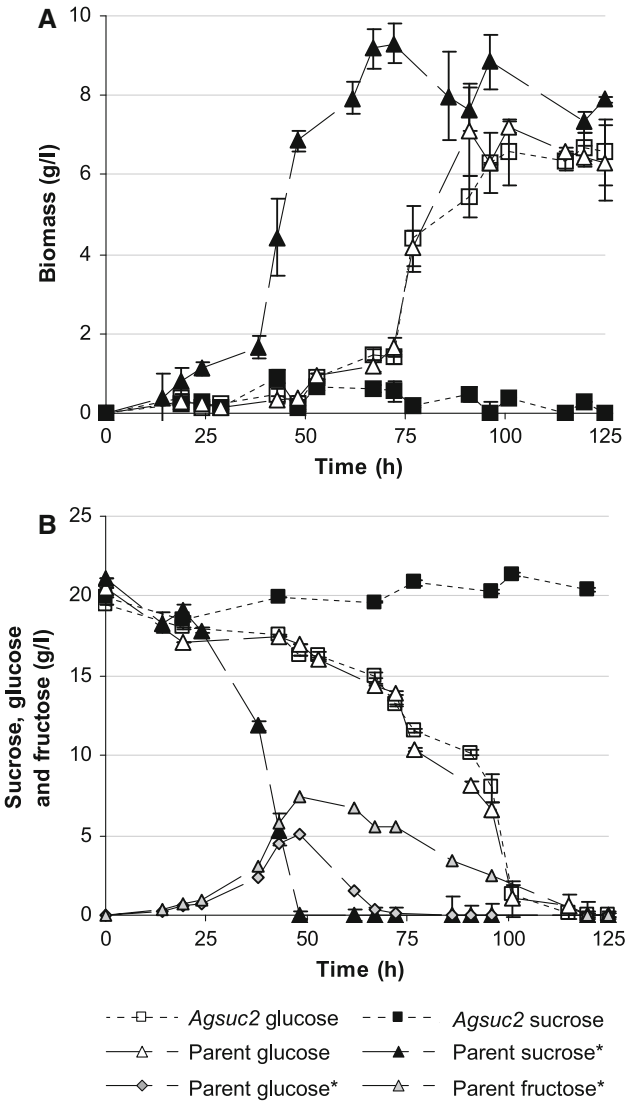

Fig. 4 Comparison of biomass production (**a**) and sugar consumption (**b**) by *A. gossypii* ATCC 10895 (parent strain, *triangles*) and the *A. gossypii Agsuc2* null mutant (*squares*) growing in 250-ml flasks containing 50 ml of SC medium with sucrose (*solid symbols*) or glucose (*open symbols*) as primary carbon source. *Error bars* represent the standard error of the mean of three independent biological replicates and where not seen were smaller than the symbol. * The parent strain growth in sucrose as the primary carbon

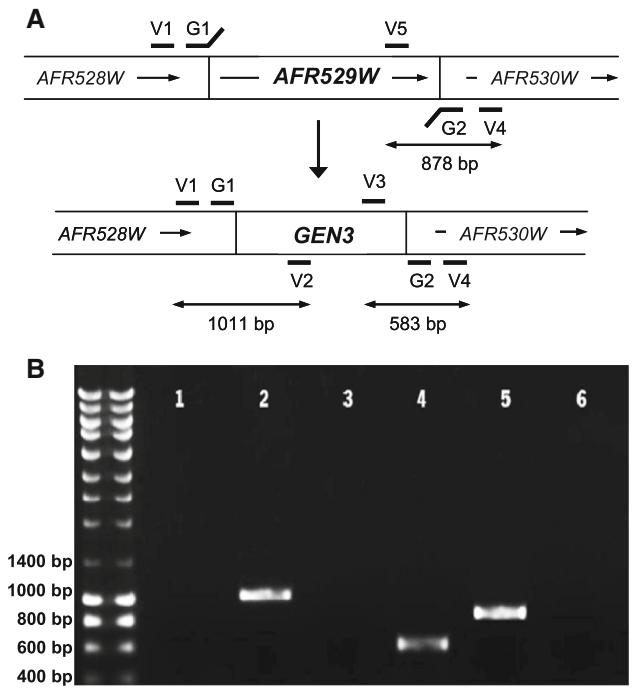

Fig. 5 Schematic representation of the *AgSUC2* gene deletion strategy (**a**) and PCR confirming the integration of the *GEN3* deletion module in the *AgSUC2* locus and complete removal of this gene from the genome of *A. gossypii* homokaryotic *Agsuc2* null mutants (**b**). **b** The *first lane* is the molecular marker (NZYDNA Ladder III, NZYTech); *lanes 1, 3, and 5* are genomic DNA from the *A. gossypii* parent strain amplified with primers V1/V2, V3/V4, and V5/V4, respectively; and *lanes 2, 4 and 6* are genomic DNA from *A. gossypii Agsuc2* mutant strain amplified with primers V1/V2, V3/V4, and V5/V4, respectively

growth on glucose was unaffected (Fig. 4). Moreover, invertase activity was not detected in either the secreted or cell-associated fractions (Fig. 6, lanes 4 and 7). Transformation of these mutants with the plasmids pTAgSUC and pTScSUC2, expressing the homologous *AgSUC2* and the heterologous *ScSUC2* genes under the control of the *S. cerevisiae PGK1* constitutive promoter, respectively, restored their ability to grow on sucrose and to secrete invertase. Invertase activity was detected in the culture filtrates of recombinant *A. gossypii* expressing both AgSuc2p and ScSuc2p. The mutants transformed with the empty vector (pMINATPS) remained unable to hydrolyze sucrose. These data demonstrate that the *AgSUC2* gene encodes all active invertase isoforms in *A. gossypii* and that it is functionally complemented by the *ScSUC2* gene. In addition, the signal peptide of the ScSuc2p was recognized by *A. gossypii* as a secretion signal.

In silico analysis of the deduced amino acid sequence of the AgSuc2p provided evidence of a potential secretion signal and a predicted cleavage site between positions 18 and 19 (SignalP 3.0 [31]). In addition, it was predicted to contain 1 putative *O*-glycosylation site (NetOGlyc 3.1 [32]) and 11 putative *N*-glycosylation sites (NetNGlyc 1.0 [33]), 7 of which are conserved in ScSuc2p (Clustal Omega [30]) (Fig. 2).

Analysis of a native gel stained for invertase activity, which had been loaded with *A. gossypii* concentrated culture filtrates, revealed two diffuse bands (Fig. 6, lanes 3 and 5), one with a molecular mass similar to that of a purified extracellular *S. cerevisiae* invertase (Fig. 6, lanes 1 and 2) and another with a lower molecular mass, indicating the existence of at least two extracellular invertase isoforms. These are most likely glycosylated, as *A. gossypii*

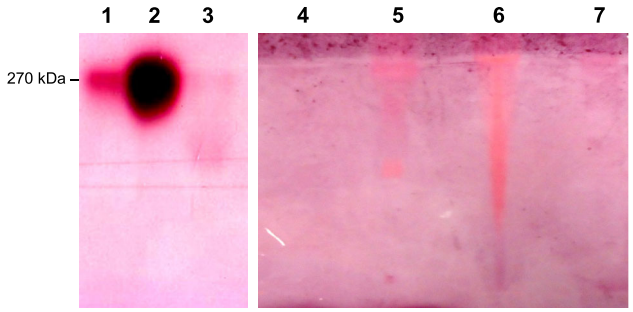

Fig. 6 Electrophoretic analyses of *A. gossypii* culture supernatants and soluble mycelial fractions in 8 % native polyacrylamide gels stained for invertase activity. *Lanes 1 and 2* 0.005 and 0.05 U of commercial invertase from *S. cereviae*, respectively. According to the manufacturer's information the molecular weight of the *S. cereviae* invertase is 270 kDa. *Lane 3 A. gossypii* culture supernatant collected from a bioreactor cultivation in defined minimal medium containing sucrose as primary carbon source after 32 h of growth and 25 times concentrated. *A. gossypii Agsuc2* null mutant culture supernatant 500 times concentrated (*lane 4*) and corresponding soluble mycelial fraction (*lane 7*) prepared after 24 h of growth in shake-flasks with AFM containing glycerol as primary carbon source. *A. gossypii* culture supernatant 500 times concentrated (*lane 5*) and corresponding soluble mycelial fraction (*lane 6*) prepared after 24 h of growth in shake-flasks with AFM containing sucrose as primary carbon source

has been previously shown to glycosylate its secreted proteins [4, 41]. The commercial invertase from *S. cerevisiae* that was used as a control produced only one band (Fig. 6, lanes 1 and 2), which according to the manufacturer's information corresponds to the 270 kDa extracellular glycosylated homodimer described by Gascón et al. [42]. Invertase from the *A. gossypii* soluble mycelial fraction appeared in the native gel as a long smeared band (Fig. 6, lane 6), suggesting the presence of other cell-wall bound and intracellular isoforms.

The existence of different invertase isoforms is not unusual in fungi. In *S. cerevisiae*, invertase occurs in two homodimeric isoforms, one intracellular and non-glycosylated (135 kDa) and other extracellular and glycosylated (270 kDa) [42]. The *S. pombe* invertase natively occurs as a dimeric (205 kDa) or multimeric (1,070–1,200 kDa) enzyme [43]. Invertases from the yeast *Arxula adeninivorans* [44] and *Rhodotorula glutinis* [45] exist as hexameric (600 kDa) and dimeric (100 kDa) structures, respectively. Invertases from *A. niger* [46] and *A. ochraceus* [14] are homodimeric proteins with apparent molecular masses of 95 kDa and 135 kDa, respectively. Invertase from *Fusarium oxysporum* culture filtrates indicated that two secreted isoforms with apparent molecular weight of 60 and 120 kDa were present [47]. High and low molecular weight isoforms (S- and F-forms differing in dimerization and glycosylation) of secreted invertase have also been described in *A. nidulans* [48]. The sugar composition of the growth media has been shown to deeply influence the different invertase isoforms created [47]. The existence of multiple isoenzymes under different growth conditions may represent a physiological advantage for microorganisms, by allowing them greater flexibility in the regulation of carbohydrate metabolism [47].

Conclusion

Based on the analysis of the *A. gossypii* genome sequence we have isolated and characterized the *AgSUC2* gene (*AFR529W* ORF) and proved that it encodes a secreted invertase that is responsible in *A. gossypii* for hydrolyzing sucrose to the readily assimilated sugars glucose and fructose. Similar to the invertases of other fungi, the *A. gossypii* invertase natively exists in more than one isoform and its synthesis is repressed by the presence of glucose in the growth medium. This provides the characterization of the second hydrolytic enzyme natively secreted by *A. gossypii*, expanding our knowledge about the protein secretion capacity of this fungus. Moreover, this work enlarges the pool of experimentally characterized *A. gossypii* ORFs.

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