Random and direct mutagenesis to enhance protein secretion in Ashbya gossypii

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Introduction

Because of their natural ability to secrete high amounts of extracellular proteins, filamentous fungi have been extensively exploited for the production of homologous and heterologous proteins. Although homologous protein production can reach the level of hundreds of grams per liter, the production levels of heterologous proteins can be several orders of magnitude lower.1,2 In the search to further improve the properties of fungi as protein producers, many strategies have been employed and optimized, including signal sequence optimization, use of strong promoters, different microorganisms,16 including mutagenesis using nitrous acid, hydroxylamine, UV radiation, transposons and EMS.17 Mutagenic procedures can be optimized in terms of type of mutagen, dose and temperature. EMS is an alkylating agent that induces point mutations by A-T transition to G-C.18 This mutagenic agent has been used to increase glucose oxidase activity,19 phytase,20 pectinase,21 catalase,19 lipase,22 laccase23 and citric acid production in Aspergillus niger,24,25 as well as to improve recombinant strains of Saccharomyces cerevisiae26 and Trichoderma viride,27 among others. Selection of a heterologous protein often represents a major bottleneck. Once a protein has been released out of the ER–Golgi system, it has to cross the cell wall. Mutations in genes involved in the construction and in the maintenance of the cell wall, such as PMR1, SEC14, EBD1, MNN9 and MNN10, have in some cases been demonstrated to lead to secreting mutants with improved secretion capabilities (such as the knockout of SEC14) which improve secretion activities.3,28

To improve the general secretion ability of the biotechnologically relevant fungus Ashbya gossypii, random mutagenesis with ethyl methane sulfonate (EMS) was performed. The selection and screening strategy followed revealed mutants with increased secretion of heterologous Trichoderma reesei endoglucanase I (EGI), native α-amylase and/or native β-glucosidase. One mutant, S456, presented 1.4- to 2-fold increases in all extracellular enzymatic activities measured, when compared with the parent strain, pointing to a global improvement in protein secretion. Three other mutants exhibited 2- to 3-fold improvements in only one (S397, B390) or two (S466) of the measured activities. A targeted genetic approach was also followed. Two homologs of the Saccharomyces cerevisiae GAS1, AgGAS1A (AGL31W) and AgGAS1B (AGL32W), were deleted from the A. gossypii genome. For both copies deletion, a new antibiotic marker cassette conferring resistance to phleomycin, BLE3, was constructed. GAS1 encodes an β-1,3-glucanosyltransferase 16 involved in cell wall assembly. Higher permeability of the cell wall was expected to increase the protein secretion capacity. However, total protein secreted to culture supernatants and secreted into Biological Engineering; Universidade do Minho; Braga, Portugal; 2VTT Technical Research Centre of Finland; Otoniä, Finland

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It is a routine practice to develop mutants through random mutagenesis. Strain improvement by random mutagenesis is a successful method, but it is mainly a trial-and-error process, involving laborious procedures. Moreover, in many cases improved performance is a black box, and the underlying mechanism is not easily identified.6 The main effect of mutagenic agents (X-rays, UV-rays, nitrous acid, dimethyl sulphamate, ethyl methane sulphonate (EMS) and acetone mustard) relies on creating a lesion or a modification in the base sequence of the DNA molecule. If this lesion is not repaired a mutation is caused.7 The nature of the mutagenic agent is such that changes are not directed exclusively at the loci which will generate beneficial change, requiring the screening of large numbers of strains for the desired phenotypes. Different methods have been used to introduce random mutations in different microorganisms,8 including mutagenesis using nitrous acid, hydroxylamine, UV radiation, transposons and EMS.9 Mutagenic procedures can be optimized in terms of type of mutagen, dose and temperature. EMS is an alkylating agent that induces point mutations by A-T transition to G-C.10 This mutagenic agent has been used to increase glucose oxidase activity,10 phytase,11 pectinase,11 catalase,11 lipase,12 laccase12 and citric acid production in Aspergillus niger,13,14 as well as to improve recombinant strains of Saccharomyces cerevisiae15 and Trichoderma viride,16 among others. Selection of a heterologous protein often represents a major bottleneck. Once a protein has been released out of the ER–Golgi system, it has to cross the cell wall. Mutations in genes involved in the construction and in the maintenance of the cell wall, such as PMR1, SEC14, EBD1, MNN9 and MNN10, have in some cases been demonstrated to lead to secreting mutants with increased secretion activities.3

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in *S. cerevisiae* and other yeast. In addition, the deletion of the cell wall cross-linking enzyme glycosphosphatidylinositol-anchored surface Gas1p led to an almost 7-fold increase in the level of human insulin-like growth factor 1 (hIGF1) in *S. cerevisiae*. In *Pichia pastoris*, deletion of the GAS1 homolog resulted in a supersecreting phenotype for the heterologous *Bacillus* subtilis lipase, but had no effect on the secretion of recombinant human trypsinogen and albumin. More recently, deletion of the *Zygosaccharomyces bailii* GAS1 homolog almost doubled the amount of recombinant *Candida* lipase and *Terremia* lipoprotein per cell and increased the extracellular enzymatic activity, but no relevant effect was observed on recombinant human IL-1b secretion.

*Ashbya gossypii* is a filamentous hemiascomycete with a natural capacity to produce riboflavin (vitamin B2), which is used for the industrial production of this vitamin. The potential of *A. gossypii* as a host for recombinant protein production was previously evaluated by expressing cellobiohydrolase I (CBHI) and endoglucanase 1 (EG1) from *Trichoderma reesei*. However, the secretion levels obtained were very low, particularly in the case of CBHI. Here, we describe the improvement of the general secretion capability of *A. gossypii*. Two approaches were used: random mutagenesis with EMS and direct mutagenesis by individual and multiple deletion from the genome of both copies of the GAS1 gene, *AgGAS1A* (*AGL351W*) and *AgGAS1B* (*AGL352W*).

**Results**

EMS treatment parameters for mutagenesis of recombinant *A. gossypii*. Since *A. gossypii* spores cluster together in groups of needle shape spores difficult to separate, treatment with EMS resulted in highly variable numbers of colonies per plate after treatment. Between 46–100% lethality was obtained when using 5–20% (v/v) EMS during 1 and 2 h of incubation at room temperature. One hundred per cent mortality was recorded after 3 h of EMS treatment. Average killing rates of 50–90% were obtained after treatment with 5% (v/v) EMS for 90 min, which were considered to be suitable for mutant screening (data not shown).

Mutant screening and characterization of putative *A. gossypii* mutants with improved secretion capacity. Primary selection of hyperproducing mutants was done based on the diameter of the clearing zone surrounding the colony on the screening media containing 0.1% (w/v) carboxymethylcellulose (CMC) and G418 (200 μg ml⁻¹). In addition to CMC, mutants were also screened on starch and tributyrin agar plates for α-amylase and lipase activity, respectively. Screening on medium containing CMC gave a fairly reliable indication of increased cellulolytic activity, whereas screening on starch and tributyrin plates was less conclusive (data not shown).

In the primary selection, approximately 1000 mutant colonies showing larger clearing zones than the control strain were isolated from the screening plates. Of these, the 39 mutants that presented the biggest haloes in the CMC plates were selected and enzyme production was determined in submerged cultures (Fig. 1). Comparison of the EGI and β-glucosidase extracellular activities between the control strain and the 39 selected mutants was made at several times after inoculation, to take into account differences in the lag phase or specific growth rate on enzyme production and/or secretion. The EGI and β-glucosidase specific activities (per gram of dry cell weight) quantified from the culture supernatants increased along the culture time reaching their maximum levels at the stationary phase (data not shown). From these 39 mutants, the most promising 5 were selected for further analysis, which showed increase in both EGI and β-glucosidase activity (mutant S436), only in EGI activity (mutants B390 and S466) or only in β-glucosidase activity (mutants B206 and S397) (Fig. 2).

The EGI, α-amylase and β-glucosidase specific activities measured from the supernatants of the selected mutants at different cultivation times are depicted in Figure 2. There were no significant (p > 0.05) differences in the specific growth rate of the mutants when compared with the parent strain. The specific growth rate varied between 0.09–0.16 h⁻¹ in AFM, which was in agreement with values reported for the recombinant *A. gossypii* VTT D-101398. Three mutants presented enhanced EGI activity (p < 0.05), with mutant B390 showing nearly a 3-fold increase and mutants S436 and S466 a 2-fold increase compared with the parent in flask culture after 63 h of growth (Fig. 2A). At this point all cultures had reached the stationary phase (data not shown).

In comparison to the control strain, mutant S466 also presented increased α-amylase activity (p < 0.01) at the deceleration phase (after 30 h and 43 h of growth) and mutant S436 presented an increase in both α-amylase and β-glucosidase activities as well (Fig. 2B and C). The mutant with the highest β-glucosidase activity was mutant S397, which produced 2-fold (p < 0.05)
more compared with the non-mutagenized strain (Fig. 2D).

Effect of \( \text{AgGAS1} \) gene deletion on recombinant protein production. For the inactivation of \( A. \text{gossypii} \) ATCC10895 \( \text{AgGAS1} \) and \( \text{AgGAS1B} \), the entire open reading frames \( \text{AGL351} \) and \( \text{AGL352} \) were replaced with the NATPS cassette flanked by 66 bp and 67 bp upstream and downstream of \( \text{AGL351} \) and \( \text{AGL352} \) sequences, to enable homologous recombination and genomic insertion using a PCR strategy. As can be seen in Figure 3, \( \text{AgGAS1} \) elimination was confirmed in three mutants. The expected size fragments (Fig. 3B) or no amplification (Fig. 3C), were obtained. The mutants did not show any visible phenotype variation in comparison to the parent strain when grown on solid or in liquid AFM with glucose (Fig. 4A and B). However, microscopically it was observed that their mycelia presented more vacuoles than the parent strain mycelia (Fig. 4E and F).

Elimination of the \( \text{AgGAS1B} \) was also accomplished, as can be seen by the expected sizes of the products obtained from the verification PCR (Fig. 3D and E). The resulting mutants exhibited an extremely slow growth rate on solid AFM medium (Fig. 4C and D) and altered morphology (Fig. 4G and H), even on plates containing 1 M sorbitol as osmotic support. Moreover, the heterokaryotic \( \text{Aggas1A} \Delta \text{gas1B} \Delta \) double mutant also presented defects in sporulation. The mycelia of the \( \text{Aggas1B} \Delta \) single mutant (Fig. 4G) presented granular cytoplasm and higher number of vacuoles than the parent strain (Fig. 4E). These were also bigger than the vacuoles observed in the mycelia from the parent strain. Similarly, intracellular granules and altered vacuoles were also visible in the heterokaryotic \( \text{Aggas1A} \Delta \text{gas1B} \Delta \) mutant (Fig. 4H), for which it was observed that a large fraction of cells underwent lysis even in the presence of an osmotic stabilizer. The extremely slow growth rates presented by the \( \text{Aggas1B} \Delta \) null mutants imposed several experimental and processing constraints, hampering the evaluation of the effect of \( \text{AgGAS1B} \) deletion on protein secretion. The \( S. \text{cerevisiae ADH1} \) terminator has been reported to display autonomous replicating sequence activity in \( A. \text{gossypii} \).38 In this study, the modification of the plasmid pMIS19, which also included the removal of the \( S. \text{cerevisiae ADH1} \) terminator sequence present, resulted in a 2-fold increase in the extracellular EGL activity, when compared with the strains expressing EGL from the original pMIS19 plasmid (VTT D-101398 strain) (Fig. 5). Therefore, the modified pMIS19 plasmid was chosen to be introduced in the \( A. \text{gossypii} \) NQ89 strain to assess the effect of the \( \text{AgGAS1A} \) deletion in the production of \( T. \text{reesei} \) EGL.
mutagenesis with the goal of increasing riboflavin production.39,40 Although there are numerous studies on the application of EMS to induce mutagenesis in other microorganisms, this is the first time that Ag. gossypii secretion mutants have been produced by means of EMS mutagenesis.

**Figure 3.** PCR confirmation of GAS1 deletion in Ag. gossypii. (A) Schematic representation with the location of the primers used for verification of AgGAS1A gene deletion and corresponding amplicon sizes. (B) PCR with primers VFW_351 and VRR_351 was made using as template lanes 1, 2 and 3 genomic DNA (gDNA) from homokaryotic transformants (predicted fragment size of 1618 bp); lane 5 parental gDNA (predicted fragment size of 1975 bp). Lanes 1, 2 and 3 gDNA from homokaryotic transformants (no amplification); and lane 5 parental gDNA (predicted fragment size of 1345 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. (C) PCR with primers VFW_351 and VRR_351 was made using as template lanes 1, 2 and 3 gDNA from homokaryotic transformants (predicted fragment size of 1618 bp); lane 5 parental gDNA (predicted fragment size of 1975 bp). Lanes 1, 2 and 3 gDNA from homokaryotic transformants (no amplification); and lane 5 parental gDNA (predicted fragment size of 1345 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. (D) Lane 1 molecular marker (NZYTech). Lanes 2, 4 and 6 parental gDNA respectively amplified with primers: V2FW_352 and VR352_RV (predicted fragment size of 1935 bp for the parent and 1692 bp when the deletion cassette has been integrated in the genome), and V3FW_352 and VR352_RV (predicted fragment size of 1345 bp for the deleted strain and no amplification in the parental strain). Lanes 1, 3, 5 and 7 correspond to gDNA from a homokaryotic AggAS1Δ transformant amplified with the same sets of primers used with the parental strain. (E) Schematic representation with the location of the primers used for verification of AgGAS1B deletion and corresponding amplicon sizes.

**Discussion**

Several mutants of Ag. gossypii have been generated using UV radiation and N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) mutagenesis with the goal of increasing riboflavin production.39,40 Although there are numerous studies on the application of EMS to induce mutagenesis in other microorganisms, this is the first time that Ag. gossypii secretion mutants have been produced by means of EMS mutagenesis.

EMS treatment was effective in generating Ag. gossypii mutants with improved extracellular enzymatic activities (Figs. 1 and 2), even though only 50–90% of killing was obtained after 90 min of incubation at room temperature with 5% (v/v) EMS. As no significant differences were observed between the growth of the parent and of the isolated mutant strains, the enhanced extracellular enzyme production by the mutants was not due to an increase in growth, but to an improvement in protein production and/or secretion. Three mutants were isolated with enhanced EGI extracellular activity (Fig. 2A). One of these mutants (S436)
also presented increased α-amylase and β-glucosidase extracellular activities, as compared with the parent strain (Figs. 2B and C), indicating that the mutation(s) affected the secretory pathway in general. Other mutant (S466) showed a 3-fold increased extra-cellular α-amylase activity as well, whereas mutant B390 only presented increased EGI activity. Additionally, one mutant was isolated which did not have improved EGI activity, but which produced more extracellular β-glucosidase (S397). In mutants B390, S397 and S466, as the mutation(s) only affected the production of some enzymes, the activity increase observed corresponds to a specific event which may not be associated with an improvement on the secretory pathway per se, but be protein specific.

Mutant S436, with 1.4- to 2-fold improved secreted activity (Fig. 2), would be useful for further development of A. gossy-pii protein production. 2-fold improvements in secreted hydro-lytic activity are generally observed for other mutants obtained by random mutagenesis. For example, using different mutagens (ethidium bromide, NNMG and UV) to treat Trichoderma sp. and Aspergillus sp. ssp., Chaud et al.44 reported 1.9- to 2.9-fold increase in cellulase production. Dillon et al.45 reported 1.5-fold more cellulase productivity in Penicillium chrysogenum than by three repeated mutagenic treatment steps with UV radiation. T. reesei TL-124 was subjected to successive mutagenic treatments to obtain mutants with 2- to 2.6-fold increased cellulase production relative to the wild type strain.46 Aspergillus sp. has been improved for cellulase production by sequential treatments with γ-radiation, UV radiation and NNMG to obtain improved mutants which produced 2- to 3-fold improvements in cellulolytic activity.41 In this regard, the range of improvement obtained here is similar to what has been obtained in other microorganisms, although secretion is low compared with e.g., Trichoderma. The low secretory ability of A. gossypii made screening for secretory mutants difficult. The difficulty of rapidly screening for cellulase hyperproducing mutants of Neurospora and Trichoderma has previously been noted.47 For A. gossypii, the haloes in agar plate assays were small and affected by the depth of agar and size of fungal colony. Germination time following mutagenesis was variable and thus colonies of different sizes needed to be compared. It was also not practical to restrict colony expansion by inclusion of agents such as Tween 20 or Triton X100, which have been used with T. reesei,48 because they inhibited A. gossypii spores germination. Nonetheless, the screening method used did allow direct selection of hyperproducing mutants of the target protein from the colonies after mutagenesis, even though false positives were also isolated (Figs. 1 and 2).

Targeted genetic modification, by deletion of the A. gossypii GAS1 homologs, was less successful than random mutagenesis. GAS1 was chosen as a target for A. gossypii strain improvement because in yeast it encodes a cross-linking enzyme known to affect the structure and permeability of the cell wall. Cell wall defective strains deleted in the GAS1 gene of S. cerevisiae,49 P. pastoris50 and Z. bailii51 have been reported as super-secreting. Deletion of GAS1 in S. cerevisiae resulted in an almost 7-fold increase in the level of human hIGF1.52 Similarly, secreted enzymatic activity was almost doubled when GAS1 was deleted in Z. bailii producing C. rugosa lipase Cel1p or Y. lipolytica protease Xep2p.53 In A. fumigatus, an increase in glycoprotein secretion has also been reported for goαΔA mutants.53 Since phylogenetically A. gossypii is closely related to S. cerevi-siae and both show similar secretion characteristics,54 deletion of its GAS1 homologs might have been expected to result in similar improvements in secretion. However, the deletion of AgGAS1A from A. gossypii genome did not cause any effect on the capacity of A. gossypii to secrete heterologous EGI (Fig. 4), or native α-amylase (data not shown). Unless, we isolated two tandem copies of the GAS1 gene in the A. gossypii genome. Here we have only tested the effect of the AgGAS1A deletion on protein secretion, because both Aggas1A and Aggas1AgoαΔA mutants exhibited severe limitations in terms of growth. Therefore, there may still be sufficient protein in the wall of the Aggas1AΔA mutant to explain the lack of improved secretion. It is worth not-ing that also in P. pastoris the disruption of GAS1 had no effect on the secretion of human trypsinogen or human serum albumin, although the amount of R. oryzae lipase released from the cells was doubled.55 Similarly, GAS1 deletion in Z. bailii did not increase human IL-1β secretion.56 Improvements in GAS1 deletion strains have been protein dependent and EGI is not a read-ily secreted protein. Altogether, this may have contributed to the lack of improvement.

The inactivation of the AgGAS1 homologs affected A. gossypii morphology and growth rate, as already reported for other GAS1-deficient organisms. Similarly to what has been observed in yeast,57 A. gossypii Aggas1A mutants exhibited severe growth retardation and abnormal cells with granular cytoplasm and big vacuoles. Moreover, the Aggas1AgoαΔA double mutant presented sporulation deficiency and a high percentage of cellular ehytes even when oomycotic support was present. In A. fumigatus, abnormal conidio genesis and slower growth were observed when GEL2, a ortholog of the GAS1 gene family, was deleted.58 These peculiar morphological alterations have been associated with altered structure and composition of the mutants cell wall.59-67 The major output of this study was one mutant with overall improved secretion capability obtained by random mutagenesis. Moreover, a new antibiotic marker cassette, BLE3, consisting of the ble′ gene from the bacterial transposon Tn5 flanked by the promoter and terminator sequences of the S. cerevisiae TEF2 gene, has also been constructed for use in A. gossypii gene dele-tions, allowing selection of plasmcid-resistant transformants. Targeted deletion of AgGAS1A had no significant effect on the overall A. gossypii secretion ability and the deletion of AgGAS1B led to defects in cellular growth, morphology and sporulation.

Materials and Methods

Strains. Recombinant A. gossypii VTT D-101398 expressing EGI from T. reesei, constructed previously,53 was used throughout this study. A. gossypii ATCC10895 (kindly provided by Prof P Philipsen from Basel) was used for gene deletion experiments.

Media. Ashbya Full Medium (AFM; 10 g l⁻¹ peptone, 10 g l⁻¹ yeast extract, 1 g l⁻¹ myo-inositol plus a carbon source) was used for growth of A. gossypii. Starch (10 g l⁻¹), carboxymethylcellulose
Figure 4. Colony and cellular morphology of A. gossypii parental and gas1 null mutant strains. From (A) to (D), A. gossypii parental and gas1 null mutant colonies are shown, which were inoculated in the middle of KM plus glucose plates containing 1 M sorbitol and grown for one week at 30°C. Panels (E) to (H) show microscope images of A. gossypii parental and gas1 null mutant mycelia and needle shaped spores harvested from the center of the colonies on the right. Arrows indicate granules and enlarged vacuoles in mutant hyphae. In panel (H), extensive cellular lysis is observed.
A. gossypii agar-solidified AFM plus G418, with glucose as carbon source, in flooding the plate with congo red, colonies were transferred to its activity on the substrate (CMC). Mutants were selected on the hydrolysis were clearly visible indicating cellulase production and 0.1% (w/v), starch (10 g l\(^{-1}\)) or trybutyrin (10 ml l\(^{-1}\)).

\[ \text{PM1519 plasmid (open circle), parent strain expressing EGI from the modified pM1519 plasmid (closed circle) and parent strain expressing EGI from the original pM1519 plasmid (closed triangle).} \]

Figure 5. Average EGI activity from supernatant of recombinant A. gossypii growing in flasks with AFM plus glucose at 30°C and 200 rpm. Activities were measured with the MULac assay, described in materials and methods. Data represent average ± standard error of the mean obtained from three independent cultures. AgGaGAS1A mutant strain (OGW) expressing EGI from the modified pM1519 plasmid (open circle), parent strain expressing EGI from the modified pM1519 plasmid (closed circle) and parent strain expressing EGI from the original pM1519 plasmid (closed triangle).

(CMC) (1 g l\(^{-1}\)) or trybutyrin (10 ml l\(^{-1}\)) were provided as carbon sources for mutants screening. Media were solidified with agar (20 g l\(^{-1}\)) and when indicated 1 M sorbitol was added to AFM sources for mutants screening. Media were solidified with agar A. gossypii cultures.

For rapid screening of EGI production, mutants and the control strain of A. gossypii were further assessed for cell growth and protein secretion. Data represent average ± standard error of the mean obtained from three independent cultures. AgGaGAS1A (AGL351W) and AGL1352W from the modified pM1519 plasmid.

**Materials and methods.**

**Mutagenesis and selection.** Mutations were induced in A. gossypii VTT D-101398\(^{36}\) with EMS. Several concentrations of EMS and different incubation periods were tested to determine a treatment suitable for 90–99% killing. Based on the results, mutagenesis was subsequently performed by diluting a spore suspension of A. gossypii ten times in sodium phosphate buffer and incubating with 5% (v/v) EMS for 90 min at room temperature, with shaking. Mutagenized spores were diluted in 0.9% (w/v) NaCl and plated on agar-solidified AFM containing 0.1% (w/v) CMC plus 200 μg ml\(^{-1}\) G418 (Sigma) in order to keep the plasmid containing the recombinant EGI. A control was treated with water instead of EMS. Colonies appeared after 3 d.

For rapid screening of EGI production, mutants and the control strain of A. gossypii were incubated for 48 h on agar medium containing 0.1% (w/v) CMC as the sole carbon source and then flooded with 1% (w/v) congo red solution according to the method of Feather and Wood.\(^{37}\) From this procedure, zones of CMC hydrolysis were clearly visible indicating cellulase production and its activity on the substrate (CMC). Mutants were selected on the basis of clearing zone size after the congo red treatment. Before flooding the plate with congo red, colonies were transferred to agar-solidified AFM plus G418, and mutants were selected in CMC hydrolysis, in order to store the putative mutants. Selected mutants and A. gossypii VTT D-101398 were further assessed for cell growth and production of extracellular enzymes in AFM containing either CMC. 0.1% (w/v), starch (10 g l\(^{-1}\)) or trybutyrin (10 ml l\(^{-1}\)).

Culture conditions. Spores were prepared by collecting 8–10 d old mycelia from agar-solidified medium, digesting with zymolase (75 mg ml\(^{-1}\)) for 2 h and washing with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol and 0.025% (v/v) Tween 20.

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml AFM with glucose and 200 μg ml\(^{-1}\) G418 (Sigma) and incubated at 30°C, 200 rpm. Flasks were inoculated with spores which had been stored at −80°C. Dry cell weight was determined as previously described.\(^{38}\)

Protein and enzymatic assays. Endoglucanase I (EGI).

Endoglucanase I (EGI) activity was assayed as described previously\(^{39}\) by using 4-methylumbelliferyl-β-D-glucopyranoside (MULac, Sigma) as substrate.

α-amylase. The production of starch degrading enzymes on agar-solidified medium was detected by staining the surface with iodine solution\(^{40}\) to visualize zones in which starch had been broken down. Amylase activity by mutants exhibiting the largest hydrolysis haloes were then tested in liquid cultures. Alpha-amylase was measured by incubating 0.5 ml mutant strain at 25°C for 3–4 min to achieve temperature equilibration, after which 0.5 ml of 1% (w/v) starch solution (at 25°C) was added. After 3 min of incubation, 1 ml dinitrosalicilic acid color reagent was added. Samples were incubated in a boiling water bath for 5 min. After cooling to room temperature, 1 ml of water was added, samples were mixed and absorbance at 540 nm read vs. a blank.

Lipase. Lipase was detected by growing the fungus on agar solidified trybutyrin AFM. Degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium.

**Protein content.** The protein concentration present in the supernatant was determined by the Bradford assay using bovine serum albumin (BSA, Sigma) as the standard.

**GAS1-homolog gene deletion from the A. gossypii genome.**

In the A. gossypii genome, GAS1 is present as a tandem gene duplication, namely AgGAS1A (AGL351W) and AgGAS1B (AGL352W). In this study, A. gossypii gas1 null mutants were created using a PCR-based, one-step gene targeting approach.\(^{41,36}\)

For individual gene deletion, the disruption cassettes containing the NATPS marker, which confers resistance to nourseothricin/lorNAT, were amplified by PCR from pUC19NATPS (Hoepfner D, personal communication), using the primers 351FW and 351RV and 352FW and 352RV (Table 1). These primers were designed to have 66 bp and 67 bp flanking regions corresponding to the 5' and 3' flanking regions of AgGAS1A and AgGAS1B, respectively. Phusion\(^{\circledR}\) High-Fidelity DNA Polymerase (Finnzymes) was used and the PCR conditions were 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 50°C for 30 sec, 72°C for 1 min and a final step of 72°C for 10 min. The amplified deletion modules were purified using the QiAquick PCR purification kit (Qiagen) and used to transform A. gossypii ATCC 10895 mycelia.
Table 1. Oligonucleotides used in this study

| Primer name | Sequence |
|-------------|----------|
| 351FW       | 5’ GTT CAG TGG ACA CCG ATT CAA ACA ATC GTG AGG TTT GGG GAG GTT TGA ATT TGG TGG AAA ATC GTA gtt acg tga ttt tac tgt gg T’ |
| 351RV       | 5’ GTA CAT TTC TTT TAA GCA AAT GTG TCA AAG CTT GAG GGC ACA ATA GAG TGC TCA AGA CAT AGG TAT Gca aca gtt ttc ctt aat cca gga T’ |
| 352FW       | 5’ TAA GTT GGG GCC TGG ATC GGA AGG TAA CAG CAG TCG CTA TAT TGG TTA ATC ATC AGT TTA TTC ATT AAG CTA CAG ctc cag cca acc gct gtt a T’ |
| 352RV       | 5’ AAA GAA CAT AGA GCT TCG CTT AAT AGA GTT AHA GAG ACG CAT GAA AAT CTA TTT TTA AAT GAA GAT TAG CCC GAC ctc cag cca acc gct gtt a T’ |
| GEN3_FW     | 5’ GCT AGG GAT AAC AGG GTA AT T’ |
| GEN3_Xhol_RV| 5’ CGG CTG GAG CAT ATG AGG CGG TCT C’ |
| StTEFp EcoRI_FW | 5’ CCG AAT TCT CCA TGA GTT TTT TTA CTA AGA G C’ |
| StTEFp NcoI_RV | 5’ TCT ATT CCA TGG TTA TTA TAG ATC ATC GAC CG C’ |
| Ble_Fw      | 5’ GCA GGT CGA CAA CCC TTA AT T’ |
| Ble_EcoRI_RV| 5’ CGG AAT TCT GAT AGT ATC CTC GCA A3’ |
| 352BleFW    | 5’ TAA GTT GGG GCC TGG ATC GGA AGG TAA CAG CAG TCG CTA TAT TGG TTA ATC ATC AGT TTA TTC ATT AAG CTA CAG ctc gtc gac acc cct taa C’ |
| 352BleRV    | 5’ AAA GAA CAT AGA GCT TCG CTT AAT AGA GTT AHA GAG ACG CAT GAA AAT CTA TTT TTA AAT GAA GAT TAG CCC GAC ctc cag cca acc gct gtt a C’ |
| V4FW_351    | 5’ CTA GTG GCA TGT CTC GAC C’ |
| V4FW_351    | 5’ GCA GTT GTG GAT GAC AAC ATG CAG C’ |
| V5IR_351    | 5’ GCA GAT GAA GTG CAC ATC TTA CTG C’ |
| V2FW_352    | 5’ CAA GAA TCC GTG CTT TCA TGG C’ |
| V3IR_352    | 5’ GTA GAC GTT GAA GCC GTC GTA GAA GTC AG C’ |
| Ver352_Rv   | 5’ GCT GGA CTT TCG TCA TAG C’ |
| Ver352_Rv   | 5’ GAT TCG TCG TCG GAT TCG T3’ |

Lower case letters indicate the sequences complementary to the template.

by electroporation, as previously described.37 Transformants were selected on AFM agar containing 100 μg ml⁻¹ clonNAT (WERNER BioAgent). The A. gossypii AgastAΔA homokaryotic strain was named A. gossypii NGQ89. As this strain was resistant to clonNAT, the cassette used for the deletion of the second AgGAS1 copy (AgGAS1B) conferred resistance to phloemycin (blΔe). Plasmid pUGEN3 was created by amplification of the GEN3 module from pGGEN3 with the primers GEN3_FW and GEN3_Xhol_RV (Table 1), which was digested with BglII and Xhol (New England Biolabs) and ligated to plUG666 digested with the same enzymes. Using the primers StTEFp_EcoRI_FW and StTEFp_NcoI_RV (Table 1), all of the pUGGEN3 sequence, with the exception of the fun1 gene, was amplified with the Phusion High-Fidelity DNA Polymerase (Finnzymes) as described above. The blΔe gene was obtained by PCR from plasmid pUG666 with the primers Ble_FW and Ble_EcoRI_RV (Table 1). Both PCR fragments were digested with EcoRI and NcoI (New England Biolabs), and ligation was performed with T4 DNA Ligase (Promega) according to the manufacturer instructions. The resulting plasmid was named pUGBLE3.

The deletion cassette BLE3 (resistance module for phloemycin) was used for the elimination of the second copy of AgGAS1, AgGAS1B, was obtained by PCR from the plasmid pUGBLE3 with the primers 352BleFW and 352BleRV (Table 1). The deletion modules were used to transform A. gossypii NGQ89 mycelia by electroporation as previously described.37 Transformants were selected on AFM agar containing 10 μg ml⁻¹ phloemycin (InviroGen).

Transformation of multineucleate mycelia leads to heterokaryotic mutants, which contain a mixture of transformed and parental nuclei. Clonal selection of homokaryotic mutants was achieved by isolating and growing single germinating spores.

Confirmation of GAS1 homolog genes deletion from the A. gossypii genome. Correct integration of the disruption cassette on the AgGAS2A region was verified by analytical PCR with the primers V2FW_355 and V3IR_355, which anneal outside the deleted gene, and V4FW_351 and V5IR_351, which anneal inside the deleted region (Table 1; Figure 3A).

The single deletion of AgGAS1B was confirmed by PCR following primers (Table 1; Figure 3E): V2FW_352 and V3IR_352, which anneal inside the deleted region; V2FW_352 and Ver352_Rv, annealing outside of the deleted region; and V2FW_352 and V3IR_NATPS, which anneal outside of the deleted region and in the middle of the NATPS module, respectively.
Microscope images were taken by a Leica DMI3000 B inverted microscope, using the Leica Application Suite (LAS) software v4.0.

Modified EGI expression plasmid construction. In order to evaluate the production of EGI by the Aga1ΔA null mutant, one isolate, NOG9, was transformed with an EGI expression plasmid, or with the empty vector, PM156<sup>*</sup> as negative control. The expression plasmid pM159<sup>*</sup> containing EGI from <i>T. reesei</i> was modified by replacing the kanMX module, where kan gene was flanked by the A. <i>oryzae</i> <i>TEF2</i> promoter and <i>S. cerevisiae</i> ADH1 terminator, with the GEN3 module, in which the kan gene is under the <i>S. cerevisiae</i> <i>TEF2</i> promoter and terminator. The kanMX cassette together with the URA3 selection marker were excised from pM159<sup>*</sup> with Bgl<sub>II</sub>-NdeI (NZYTech) and substituted with the GEN3 module obtained from <i>gcn3</i><sup>Δ</sup><i>Δ</i> <i>Δ</i> strain with the same enzyme. This plasmid is referred here as modified pM159.<ref>
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