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# A CRISPR/Cas9-based multicopy integration system for protein production in *Aspergillus niger*

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The filamentous fungus *Aspergillus niger* is well known for its high protein secretion capacity and a preferred host for homologous and heterologous protein production. To improve the protein production capacity of *A. niger* even further, a set of dedicated protein production strains was made containing up to 10 glucoamylase landing sites (GLSs) at predetermined sites in the genome. These GLSs replace genes encoding enzymes abundantly present or encoding unwanted functions. Each GLS contains the promotor and terminator region of the glucoamylase gene (*glaA*), one of the highest expressed genes in *A. niger*. Integrating multiple gene copies, often realized by random integration, is known to boost protein production yields. In our approach the GLSs allow for rapid targeted gene replacement using CRISPR/Cas9-mediated genome editing. By introducing the same or different unique DNA sequences (dubbed KORE sequences) in each GLS and designing Cas9-compatible single guide RNAs, one is able to select at which GLS integration of a target gene occurs. In this way a set of identical strains with different copy numbers of the gene of interest can be easily and rapidly made to compare protein production levels. As an illustration of its potential, we successfully used the expression platform to generate multicopy *A. niger* strains producing the *Penicillium expansum* PatE::6xHis protein catalysing the final step in patulin biosynthesis. The *A. niger* strain expressing 10 copies of the *patE::6xHis* expression cassette produced about 70 $\mu g \cdot mL^{-1}$ PatE protein in the culture medium with a purity just under 90%.

## Introduction

In line with their saprophytic lifestyle, many filamentous fungi present in the soil have the potential to degrade and grow on a variety of plant cell polysaccharides such as starch, cellulose, xylans and pectin. They do so by secreting enzymes (amylases, cellulases, xylanases and pectinases) capable of hydrolysing these polysaccharides [1]. The ability of *Aspergillus niger* to grow on starch and the secretion capacity of *A. niger* of especially starch degrading enzymes, such as glucoamylase, has stimulated the development of *A. niger* as a cell factory for (recombinant) enzyme production [2–5].

The glucoamylase gene is one of the highest expressed genes on starch and degradation products of starch (maltodextrins, maltose and glucose) and is therefore also frequently used for the expression of

**Abbreviations**
CRISPR, clustered regularly interspaced short palindromic repeat; GlaA, glucoamylase A; GLS, glucoamylase landing site; KORE, knock out restoration site; Pat, patulin; sgRNA, single guide RNA.

(recombinant) proteins [5–7]. The expression of the glucoamylase gene is controlled and highly induced by starch, maltodextrins and maltose [8,9]. Its induction is dependent on the AmyR transcription factor [9]. The *A. niger glaA* gene is also highly expressed on glucose indicating that the expression of *glaA* is not strongly repressed by glucose repression mechanisms [10]. This is in contrast to what has been reported for *Aspergillus nidulans* and *Aspergillus oryzae* [11,12].

The presence of multiple copies of the glucoamylase gene in the genome has been shown to increase glucoamylase activity [13] and can therefore also improve expression of recombinant proteins. For efficient protein production it is desirable that the number of background proteins on culture broth is reduced. Abundantly present extracellular proteins in *A. niger* include starch degrading enzymes (glucoamylase, amylases and alpha-glucosidase) as well as aspartic proteases (peptidase A and peptidase B) [14–16]. Finally, efficient recovery of (recombinant) proteins from the spend medium requires low protease activity to prevent their degradation. The PrtT transcription factor is a well-known transcriptional activator for protease genes. Deletion of *prtT* in combination with preventing acidification of the medium are common approaches to reduce protease activity [17–19]. Advanced genome editing tools, such as CRISPR/Cas9-mediated genome editing, have been developed for *A. niger* [20–22] allowing new and rapid approaches for strain engineering [5,23]. In this study, the CRISPR/Cas9-mediated genome editing tool was used to create strains containing multiple copies of genes of interest by multiplex genome editing. A similar CRISPR/Cas9-mediated genome editing system was recently reported in *A. oryzae* in which two copies of a gene of interest were integrated by a single transformation to two different loci [24].

To construct an *A. niger* strain suitable for protein production three things should be taken into consideration. These considerations include: (a) presence of multiple copies of the genes of interest, (b) low background levels of extracellular proteins other than the protein of interest, and (c) low extracellular protease activity. Based on these considerations target genes for deletion were selected and the coding regions of the selected genes were deleted and replaced by a glucoamylase landing site (GLS). These GLSs contain the promotor and terminator regions of the glucoamylase gene (*glaA*), and a unique single guide RNA (sgRNA) sequence (KORE sequence) and allow efficient integration of the gene of interest at predetermined sites in the *A. niger* genome via CRISPR/Cas9-mediated genome editing. Several KORE sequences were initially designed for complementation studies to reintroduce genes after generating a gene knock out (Knock Out REstoration site) and are based on the sgRNA used to target the multicopper oxidase involved in pigment formation in spores (*brnA*; NRRL3_01040; An14g05370) [22,25].

As a proof of principle to illustrate the potential of this CRISPR/Cas9-based protein expression platform in *A. niger*, we demonstrate here the heterologous expression of the *patE* gene from *Penicillium expansum*. The *patE* gene is part of the patulin producing biosynthetic gene cluster in *P. expansum* and the *patE* gene encodes the extracellular patulin synthase. Patulin is a mycotoxin with strong antimicrobial activity towards bacteria and fungi [26]. PatE is a flavin-dependent enzyme that belongs to the glucose-methanol-choline (GMC) oxidoreductase family [27]. Detailed structural and biochemical characterization of the enzyme has been lacking thus far. This led us to produce the PatE enzyme recombinantly adding a His-tag to the C-terminus of the protein to enable purification of the enzyme as described in more detail [28]. To the best of our knowledge, expression and purification of extracellularly produced His-tagged proteins is scarcely reported and the FaeB protein of *A. niger* is the only other example in which a His-tagged protein was successfully purified from *A. niger* medium [29].

In this paper, we present the construction steps of a modified *A. niger* strain ultimately containing deletions of 12 genes and introduction of 10 landing sites. These landing sites were used to generate multi-copy strains containing 4, 7 or 10 copies of the *patE* gene, respectively. Successful copy-number dependent production of the PatE::6xHis-tagged protein was confirmed by SDS/PAGE analysis and western blot analysis.

## Results and Discussion

### Construction of an *A. niger* strain with 10 landing sites for the targeted integration of genes of interest

To build an *A. niger* host strain suitable for recombinant protein production, a CRISPR/Cas9-based approach was designed to create landing sites for the integration of a gene of interest at specific locations in the *A. niger* genome. The general approach is to create landing sites with a first round of iterative CRISPR/Cas9-based transformations and to use another round of CRISPR/Cas9-mediated genome editing to fill these landing sites with the gene of interest (Fig. 1). The promoter selected to drive expression of the gene of interest is the glucoamylase promoter (*PglaA*). The 10 landing sites selected

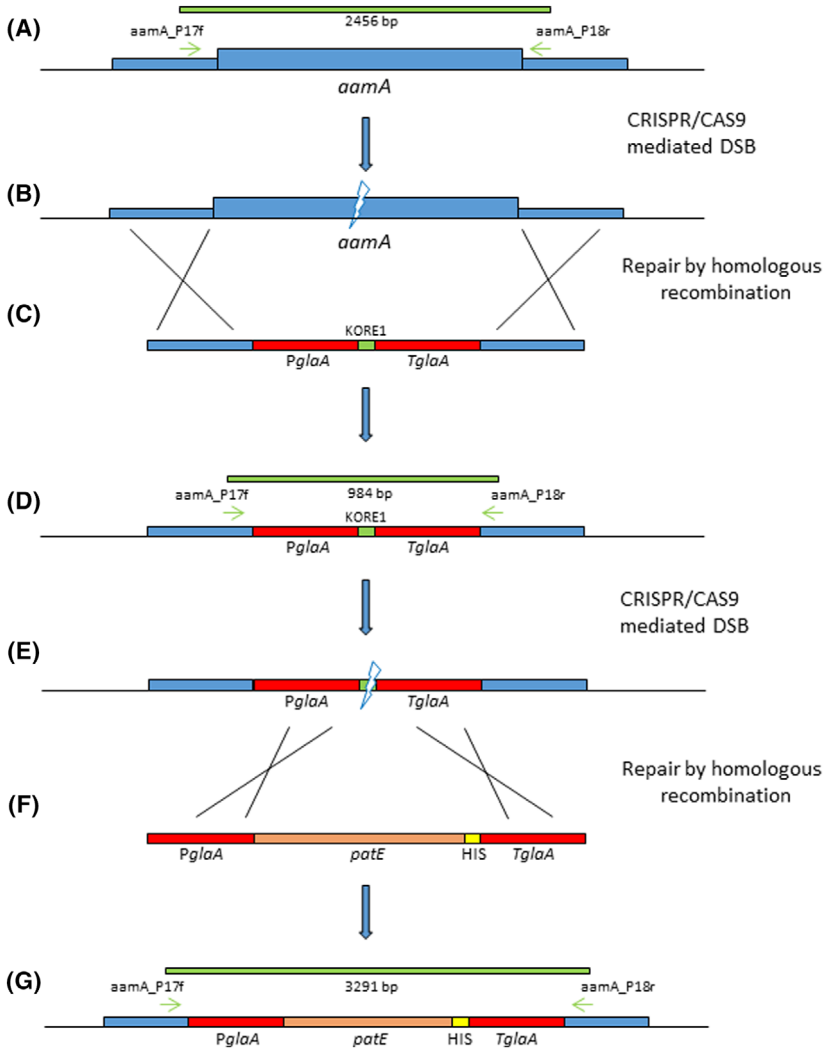



**Fig. 1.** Schematic overview of the general approach to construct a landing site (A–D) and subsequent filling of the landing site with the gene of interest (*patE*) (E–G). The *aamA* locus is shown as an example. The landing site constructions and integration of the *patE* gene were verified by diagnostic PCR and the length of the expected products are indicated with the green bars (for other genes see Table 1). (A) Genomic locus of *aamA*; (B) Using a specific sgRNA, a double strand break is created; (C) repair fragment consisting of 5′- and 3′-flanks of *aamA* and the *PglaA* and *TglaA* regions separated by a KORE sequence; (D) integration of the landing site at the *aamA* locus via homologous recombination; (E) A specific KORE1 sgRNA is used to create a double strand break; (F) repair fragment consisting of the *PglaA* and *TglaA* sequences and the gene of interest (*patE*); (G) integration of the *patE* gene at the *aamA* locus via homologous recombination.

correspond to highly expressed genes encoding starch degrading enzymes (*glaA, aamA, amyA* and *agdA*), two protease encoding genes (*pepA* and *pepB)*, the protease regulatory gene (*prtT*), a gene involved in acidification of the medium (*oahA*) and the glucose oxidase gene (*goxC*) (Table 1). Each gene was deleted using CRISPR/Cas9-based marker-free genome editing in an iterative way to be replaced by a so-called glucoamylase landing site (GLS). Three of the target genes (*agdA*, *amyA* and *prtT*) are located next to each other on the genome and these three genes were simultaneously deleted and replaced by a single landing site. Each landing site consists of part of the glucoamylase promoter (*PglaA)* and the glucoamylase terminator (*TglaA*) region. The *PglaA* and *TglaA* fragments are separated by a piece of DNA (dubbed KORE sequences) for which specific sgRNAs were designed. In total five such different KORE sequences with corresponding sgRNAs were designed. The KORE sequences are based on a *brnA* specific sgRNA, in which the 20 bp-long target-complementary CRISPR RNA (crRNA) sequence has been changed to make the sgRNAs specific and unique in the *A. niger* genome. For each KORE sequence (KORE1-5) a sgRNA (sgRNA_KORE1-5) was cloned in the sgRNA/CRISPR expression cassette (pFC332-based). Starting with a *ku70* deleted strain in the N400 (NRRL3) background, the strain lineage was constructed as shown in Table 2 and Fig. S1. Strain MA966.2, which contains 10 landing sites (Table 1), was constructed in eight consecutive transformations and used for generating a strain with four copies of *patE* (see below). To facilitate the integration of gene expression cassettes and to be more flexible in controlling the number of integrations, certain KORE sequences (KORE sequences in *pepA*, *pepB* and

**Table 1.** Overview of the 10 landing sites at the 10 selected loci.

| | | | | KORE sites at LS | | Predicted sizes PCR product at LS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LS | NRRL3_ID | Gene name | Description | MA966.2 | MA1029.4 | MA612.27 | MA966.2 | MA1029.4 | MA1005.4 4× *patE::6xHis* | MA1044.5 7× *patE::6xHis* | MA1049.9 10× *patE::6xHis* |
| #1 | NRRL3_08300 | *glaA* | Glucoamylase | 1 | 1 | 3486 | 948 | 948 | 948 | 948 | 3333* |
| #2 | NRRL3_09875 | *aamA* | Acid amylase | 1 | 1 | 2456 | 906 | 906 | 906 | 906 | 3291* |
| #3 | NRRL3_07700 | *agdA* | Alpha-glucosidase | 1 | 1 | 9711 | 950 | 950 | 950 | 950 | 3335* |
| | NRRL3_07699 | *amyA* | Amylase | | | | | | | | |
| | NRRL3_07698 | *prtT* | Protease regulator | | | | | | | | |
| #4 | NRRL3_00987 | *pepA* | Aspartic protease | 1 | 4 | 1794 | 1262 | 1006 | 1262 | 3391* | 3391* |
| #5 | NRRL3_01627 | *pepB* | Aspartic protease | 1 | 4 | 1342 | 1308 | 938 | 1308 | 3323* | 3323* |
| #6 | NRRL3_01610 | *pepN* | Aspartic peptidase | 1 + 2 | 5 | 2199 | 1485 | 964 | 3847* | 3349* | 3349* |
| #7 | NRRL3_10267 | – | Aspartic peptidase | 1 + 2 | 1 + 2 | 1715 | 1058 | 1058 | 3316* | 3316* | 3316* |
| #8 | NRRL3_02841 | *goxC* | Glucose oxidase | 1 + 3 | 1 + 3 | 2191 | 898 | 898 | 3260* | 3260* | 3260* |
| #9 | NRRL3_06354 | *oahA* | Oxaloacetate hydrolase | 1 + 3 | 1 + 3 | 1540 | 1026 | 1026 | 3388* | 3388* | 3388* |
| #10 | NRRL3_06629 | – | Carboxypeptidase | 1 + 2 | 1 + 2 | 2568 | 1218 | 1218 | 3580* | 3580* | 3580* |

Asterisk indicates the size of the PCR fragement after integration of the patE::6xHis construct.

*pepN*) were altered as described in the Materials and methods section to give strain MA1029.4. *A. niger* strain MA1029.4 was used to generate strains with 7 and 10 copies of *patE* (see below). The proper integration of the landing sites at the different loci was confirmed by diagnostic PCR as shown in Fig. 2. The sizes of the predicted PCR products are given in Table 1. The results of the diagnostic PCRs show that we successfully integrated landing sites at the 10 different loci. Deletion of the genes encoding the major starch degrading enzymes (glucoamylase (GlaA), acid amylase (AamA), amylase (AmyA) and alpha-glycosidase A (AgdA)) caused a growth reduction on plates containing starch, maltodextrin or maltose as a carbon source, but growth on glucose, fructose, xylan and Avicel (cellulose) was not affected (Fig. 3). Since the glucoamylase gene is strongly expressed by glucose, the expression of the gene of interest from the *glaA* promoter was achieved by cultivations using glucose as a carbon source.

## Construction of *A. niger* strains with different copy numbers of the *P. expansum patE* gene

To express the *P. expansum patE* gene in the described *A. niger* strains, a *patE* expression cassette was constructed. The *patE* gene was PCR amplified and fused by fusion PCR to the glucoamylase promoter (634 bp) and glucoamylase terminator region (547 bp). The PatE protein (628 amino acids long) is predicted to be secreted and the original *P. expansum* signal sequence (amino acids 1–19) was used for expression in *A. niger*. Furthermore, a 6xHis-tag was added to the C-terminus of the protein for protein purification. The expression cassette (*PglaA-patE::6xHis-TglaA*) was cloned in pJet2.1 and sequenced to confirm that there were no PCR errors. The expression cassette was excised from the pJet2.1 plasmid by *Pme*I digestion and purified from agarose gel before transformation.

The different KORE sites in the host strains (MA966.2 and MA1029.4) allow the controlled integration of the *patE* expression cassette by using different KORE sgRNAs. In the course of our experiments, we noticed that the maximal number of CRISPR/Cas9-induced double strand breaks (DSBs) followed by successful repair by the donor DNA was around five. Transformation of KORE sgRNA plasmid or combination of KORE plasmids that are expected to give more than five DSBs, resulted in very low transformation frequencies indicating that not all DSBs could be repaired efficiently enough leading to cell death. We also noticed that by trying to introduce five or more copies at the same time, not all the landing sites were filled. We therefore perform routinely

**Table 2.** Strains used in this study.

| Strain name | Genotype | Reference |
|---|---|---|
| N400 | wild-type NRRL3; ATCC9092; CBS120.49 | [32] |
| MA612.27 | *kusA::DR-amdS-DR* in N400 | [33] |
| MA950.1 | Δ*glaA::PglaA-KORE1-TglaA* in MA612.27 | This study |
| MA953.1 | Δ*aamA::PglaA-KORE1-TglaA* | This study |
| | Δ*agdA-amyA-prtT::PglaA-KORE1-TglaA* in MA950.1 | |
| MA955.1 | Δ*pepA::PglaA-KORE1-TglaA* in MA953.1 | This study |
| MA956.2 | Δ*pepB::PglaA-KORE1-TglaA* in MA955.1 | This study |
| MA960.1 | Δ*pepN::PglaA-KORE1 + 2-TglaA* in MA956.2 | This study |
| MA962.2 | Δ*NRRL3_10267::PglaA-KORE1 + 2-TglaA* in MA960.1 | This study |
| MA965.1 | Δ*goxC::PglaA-KORE1 + 3-TglaA* | This study |
| | Δ*oahA::PglaA-KORE1 + 3-TglaA* in MA962.2 | |
| MA966.2 | Δ*NRRL3_06629::PglaA-KORE1 + 2-TglaA* in MA965.1 | This study |
| MA1005.4 | Δ*pepN::PglaA-patE::6xHis-TglaA* | This study |
| | Δ*goxC::PglaA-patE::6xHis-TglaA* | |
| | Δ*oahA::PglaA-patE::6xHis-TglaA* | |
| | Δ*NRRL3_06629::PglaA-patE::6xHis-TglaA* in MA966.2 | |
| MA1029.4 | Δ*pepA::PglaA-KORE4-TglaA* | This study |
| | Δ*pepB::PglaA-KORE5-TglaA* | |
| | Δ*pepN::PglaA-KORE5-TglaA* in MA966.2 | |
| MA1037.6 | Δ*NRRL3_10267::PglaA-patE::6xHis-TglaA* | This study |
| | Δ*goxC::PglaA-patE::6xHis-TglaA* | |
| | Δ*oahA::PglaA-patE::6xHis-TglaA* | |
| | Δ*NRRL3_06629::PglaA-patE::6xHis-TglaA* in MA1029.4 | |
| MA1044.5 | Δ*pepA::PglaA-patE::6xHis-TglaA* | This study |
| | Δ*pepB::PglaA-patE::6xHis-TglaA* | |
| | Δ*pepN::PglaA-patE::6xHis-TglaA* in MA1037.6 | |
| MA1049.9 | Δ*glaA::PglaA-patE::6xHis-TglaA* | This study |
| | Δ*aamA::PglaA-patE::6xHis-TglaA* | |
| | Δ*agdA-amyA-prtT::PglaA-patE::6xHis-TglaA* in MA1044.5 | |

transformations in which we generate two up to four DSBs which are rather efficiently filled with the gene of interest in a single transformation round. Usually, integration of a gene of interest at four landing sites occurs with an efficiency of about 20–40%. Efficiencies go up significantly if less DSBs are introduced in a single transformation. It should be noted that in our hands these efficiencies vary and are prone to day-by-day variations and are depending on the efficiency of the KORE sgRNA. To determine whether an intended integration of the *patE* expression cassette occurred, usually up to 10 transformants are purified and allowed to lose the CRISPR/Cas9 sgRNA plasmid by growing the strain without hygromycin. Strains that had lost the hygromycin resistant marker (normally around 80% of the transformants) were cultured for genomic DNA isolation and diagnostic PCRs were performed to monitor the integration. As described above, for each landing site, a pair of diagnostic PCR primers was designed. By performing iterative transformations *A. niger* strains were constructed that contain 4, 7, or 10 copies of the *P. expansum patE* gene (Fig. 4, Table 2 and Fig. S1). Note that the integration of *patE::6xHis* construct at the *NRRL3_10267* locus in MA1005.4 could not be confirmed by diagnostic PCR. The absence of the PCR fragment in MA1005.4 suggests that the landing site is altered, but in an unknown way. Integration of the *patE::6xHis* construct at the *NRRL3_10267* locus in the MA1029.4 background strains was as expected.

## Analysis of PatE production in *A. niger* strains carrying different copy numbers of the *patE* expression cassette

To examine the production of PatE in strains containing increasing numbers of *patE::6xHis* expression cassettes, strains were grown in shake flask cultures and medium samples were taken after 24, 48, 72 and 96 h of cultivation. For comparison, the parental strains (MA966.2 and MA1029.4) were also cultivated under the same growth conditions. For each strain and for each time point, medium samples were loaded onto SDS/PAGE gel and proteins were stained with Sypro Ruby (Fig. 5A). Untransformed parental strains give a

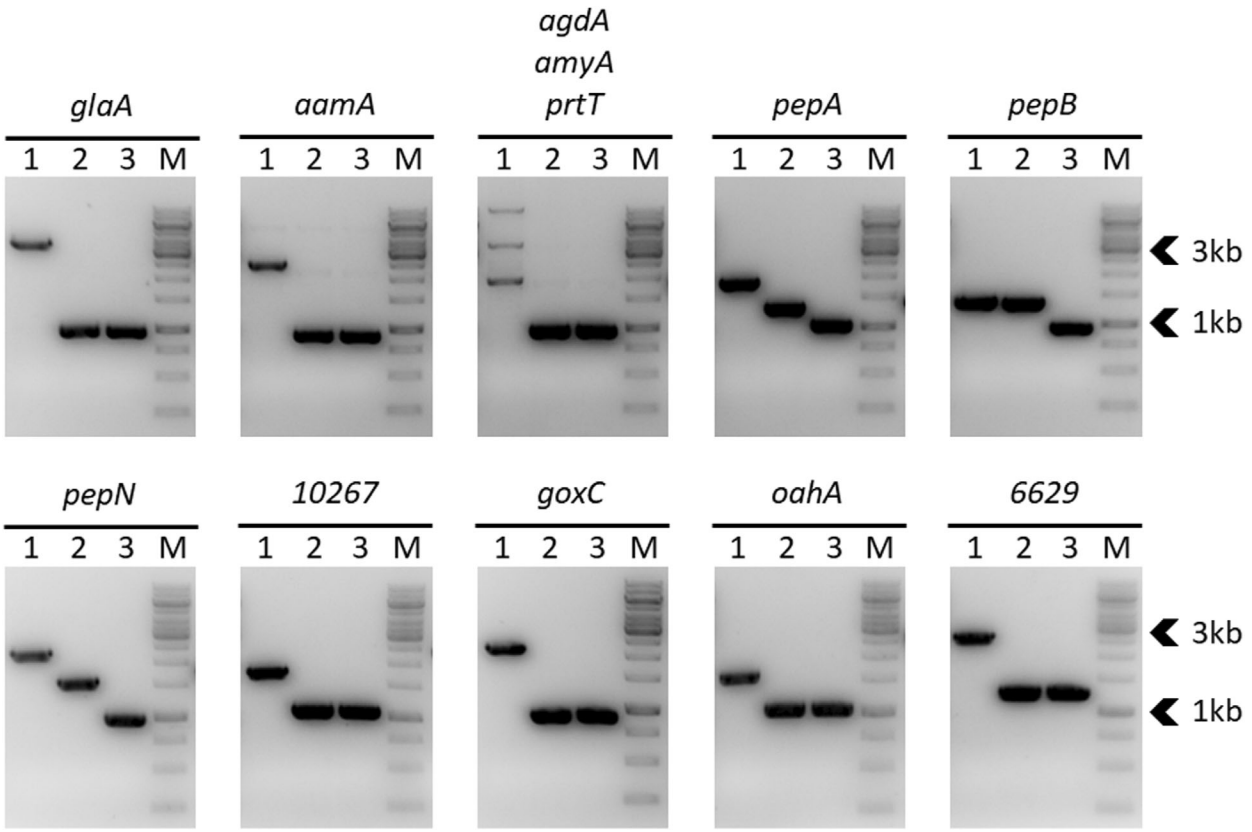


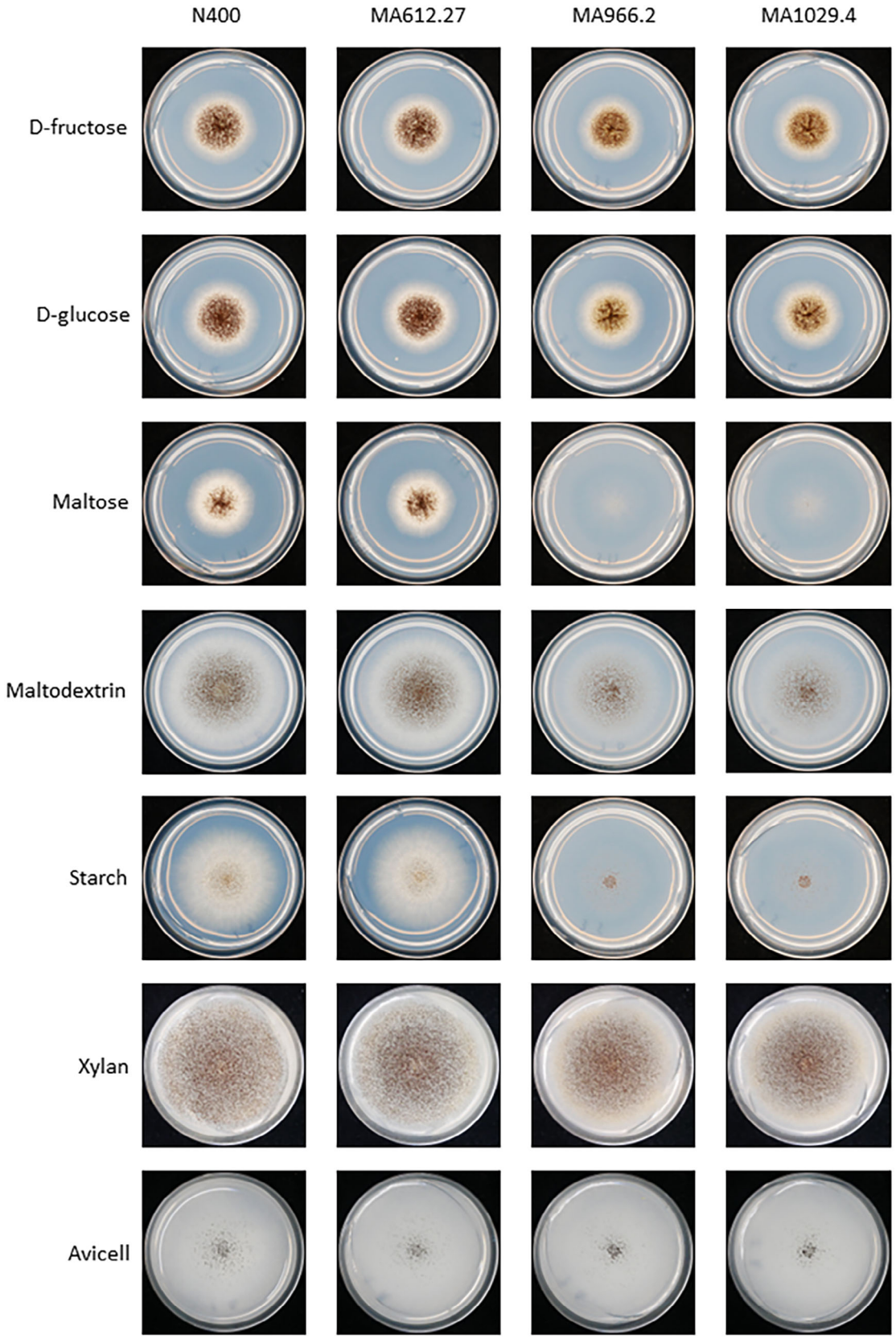

**Fig. 2.** Diagnostic PCR to verify proper integration of the landing site in 10 target genes. The predicted size of diagnostic PCRs for the 10 genes are listed in Table 1. For each gene, three diagnostic PCRs were performed on the parental strain (1), MA966.2 (2) and MA1029.4 (3). M, molecular weight marker. 1 and 3 kb are indicated by the arrows.

similar pattern characterized by relatively low levels of protein detected in the medium after 24 and 48 h of growth and a pattern of ~ 10 protein bands that become more clearly visible in the samples of the 72 and 96 h cultivation time points. The protein patterns of the strains containing the *patE::6xHis* expression construct clearly contained an additional protein with an estimated molecular weight between 80 and 110 kDa. The protein does not run as a distinct band on gel, indicating that the protein is heterogeneously glycosylated. The predicted MW of the mature protein is 67.1 kDa (including the His-tag). The protein contains nine potential N-glycosylation sites (N-X-S/T) and numerous serine and threonine residues known to be targets for O-glycosylation. Treatment of the purified PatE::6xHis protein with EndoH to remove the N-chains lowered its molecular weight from 80–110 to 70–100 kDa [28], indicating that the protein is N-glycosylated and probably also heterogeneously O-glycosylated.

Western blot analysis on the various medium samples using a His-tag specific antibody showed that the His-tag antibody recognizes a protein between 80 and 110 kDa corresponding to the PatE protein (Fig. 5B). No PatE::6xHis protein was detected in the 24 h time point sample and increasing amounts were detected at the 48 and 72 h time points. Levels of PatE::6xHis at the 96 h time point were similar or slightly less compared to the 72 h time points in all three strains. The PatE::6xHis protein expressed in the 4× *patE::6xHis* strain was successfully purified by Ni-Sepharose affinity chromatography and used for enzymatic characterization and crystallization [28].

Based on the Sypro Ruby staining, the highest level of PatE::6xHis was observed in the strain containing 10 copies of the *patE::6xHis* construct in the medium

**Fig. 3.** Growth analysis of parental strains N400 and MA612.27 compared to 10-fold deletion mutants MA966.2 and MA1029.4. Strains were grown for 4 days on D-fructose, D-glucose, maltose, maltodextrin and starch and 5 days on xylan and Avicel minimal medium with 1% (w/v) carbon source as indicated before the picture of the colony was taken.



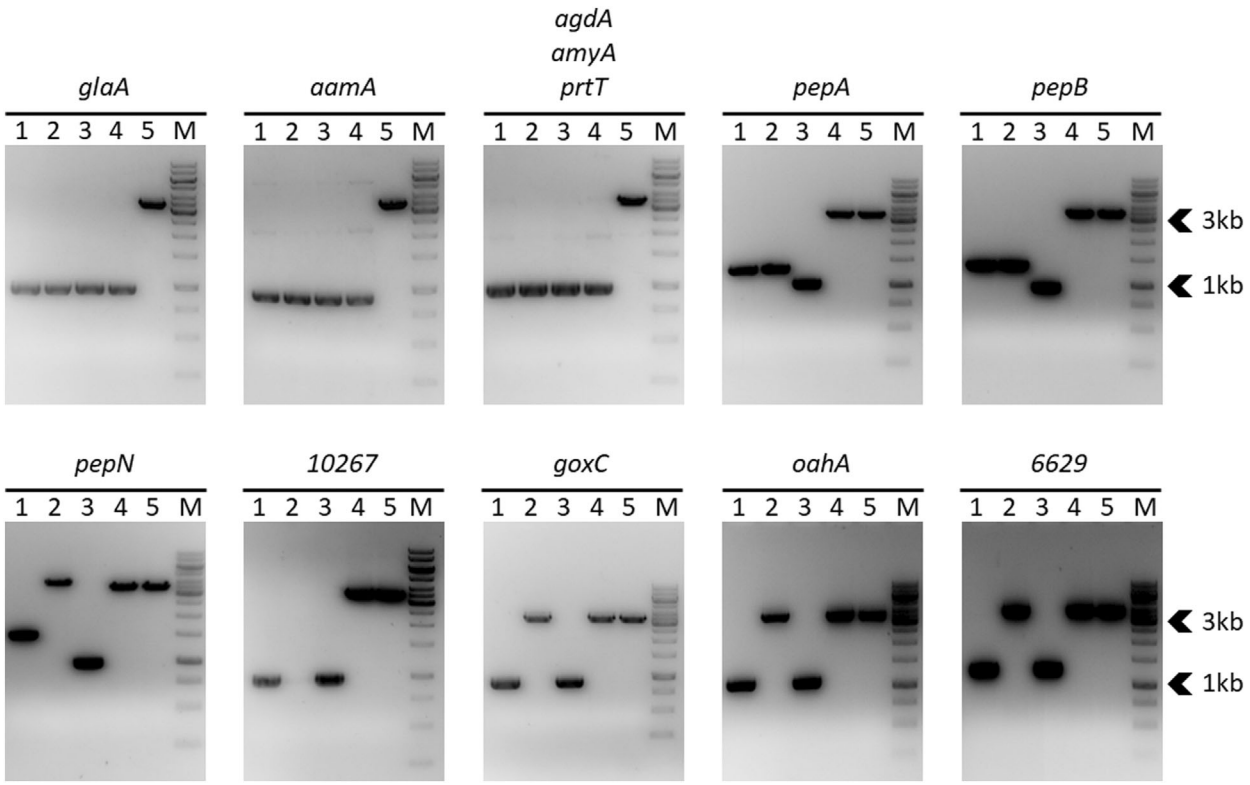



**Fig. 4.** Diagnostic PCR to verify proper integration of the *PglaA-patE::6xHis-TglaA* expression cassette in 10 landing sites. The predicted size of diagnostic PCRs for the 10 landing sites are listed in Table 1. For each gene, five diagnostic PCRs were performed on MA966.2 (1), MA1005.4 (4× *patE::6xHis*) (2), MA1029.4 (3), MA1044.5 (7× *patE::6xHis*) (4) MA1049.9 (10× *patE::6xHis*) (5). M, molecular weight marker. 1 and 3 kb are indicated by the arrows.

at the 72 h time point. The amount of protein in the 72 h medium sample collected from all transformants was determined by a Bradford assay (Fig. 5C). Protein levels in both parental strains (MA966.2 and MA1029.4) were relatively low (around 10 $\mu g \cdot mL^{-1}$) but increased to 30, 40 and 70 $\mu g \cdot mL^{-1}$ in the strain containing 4, 7 and 10 copies of the *patE::6xHis* gene, respectively. The results show that the amount of PatE::6xHis protein is correlated with copy number. The results also show that almost 90% of the protein produced at the 72 h time point in the strain containing 10 copies of the *patE::6xHis* construct, is PatE::6x-His protein. It was also tested whether the copy number of the *patE* gene impacted growth. Growth of the parental strains (N400, MA612.27, MA966.2 and MA1029.4) on either minimal medium with fructose (non-inducing condition) and glucose (inducing condition) was compared to the growth of *patE* overexpression strains (MA1005.4, MA1037.6, MA1044.5 and MA1049.9 that contain 4, 4, 7 and 10 copies of *patE*). As shown in Fig. 6 the radial growth of the *patE* overexpression strain was highly similar compared to the parental strains. A slight reduction in conidiation was observed in the 7× and 10× *patE* overexpression strains on glucose and to a lesser extent on fructose indicating that the high expression of *patE* has a small impact on growth and/or development.

In conclusion, in this study, we present an attractive strategy to efficiently produce a heterologous protein in *A. niger*. By reducing the number of background proteins and deleting the major extracellular proteases, we have generated an initial strain useful as a starting point for successful protein production of a protein of interest. The expression and presence of possible harmful proteases that would break down the protein of interest was further reduced by deleting the gene encoding the major protease transcriptional activator (*prtT*) and the oxaloacetate hydrolase encoding gene (*oahA*), thereby preventing acidification and consequently avoiding the induction of proteases by low pH via the wide domain PacC regulatory system. By deleting *goxC* we also reduced the oxidase activity which makes this particular host also suitable for the screening of fungal oxidases like e.g. the *P. expansum* PatE protein. We also show that the *A. niger* based expression platform is suitable to express secreted proteins with a His-tag. This

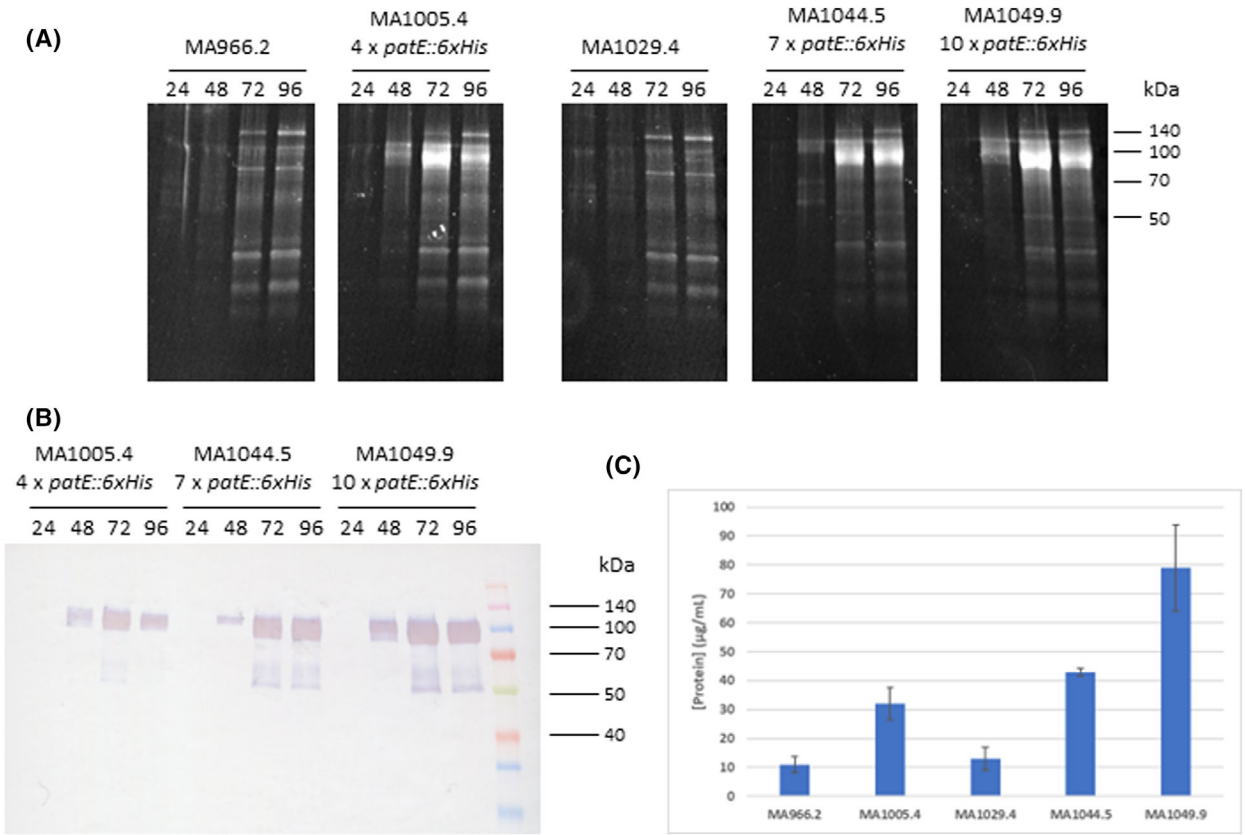



**Fig. 5.** Protein analysis of medium samples of *patE::6xHis* expressing strains. (A) Medium samples (equivalent to 8 µL of culture medium) from the parental strains (MA966.2 and MA1029.4) and strains carrying four *patE::6xHis* copies (MA1005.4), seven *patE::6xHis* copies (MA1044.5) or 10 *patE::6xHis* copies (MA1049.9) were analysed by SDS/PAGE followed by Sypro Ruby staining. (B) Western blot analysis of the strains carrying four *patE::6xHis* copies (MA1005.4), seven *patE::6xHis* copies (MA1044.5) or 10 *patE::6xHis* copies (MA1049.9). (C) Extracellular protein levels in parental strains (MA966.2 and MA1029.4) and strains carrying four *patE::6xHis* copies (MA1005.4), seven *patE::6xHis* copies (MA1044.5) or 10 *patE::6xHis* copies (MA1049.9). All values are means from measurements in triplicates from three biological experiments. The error bars indicate standard deviations.

*A. niger* based expression platform is an alternative for previously developed expression systems like *Escherichia coli* [30] or *Pichia pastoris* [31]. Furthermore, the expression system has the potential to design additional tailor-made approaches for improving production of proteins of interest, for example by further genetic modification of the proteolytic and secretion system of *A. niger*. For industrial strain improvement our strategy offers still another huge advantage as it reduces not only time and costs to construct the strain lineage but also in the registration procedures as the sites of integration are controlled.

## Materials and methods

### Strains and growth conditions

The *A. niger* strains used in this study are listed in Table 2. All strains are derived from the N400 (NRRL3) *A. niger* wild-type strain [32,33]. A Δ*ku70* derivate strain in N400 was previously constructed and used in this study [33]. Strains were grown in liquid or solidified (containing 1.5% (w/v) Scharlau agar) minimal medium (MM) or in complete medium (CM) as described [34]. Mycelial growth assays were performed on MM plates, supplemented with 1% (w/v) carbon source. D-Fructose (F2543; Sigma-Aldrich, Zwijndrecht, the Netherlands), D-glucose (50-99-7; VWR International, Amsterdam, the Netherlands) and maltose (M5885; Sigma-Aldrich) were added after autoclaving from sterile stock solutions while the polymeric substrates (soluble starch (1.01253; Merck, Amsterdam, the Netherlands), maltodextrin (D-2006; Sigma), Avicel PH101 (11365; Fluka, Zwijndrecht, the Netherlands), and xylan (4414.4; Roth, Karlsruhe, Germany)) were added before autoclaving the medium. Growth of strains was assayed by point inoculation of 5 µL myracloth filtered conidium suspension ($2 \times 10^5$ conidia·mL$^{-1}$) in the centre of a MM medium containing plate and incubated for 4–10 days in the dark at 30 °C. *E. coli* DH5α was used for plasmid

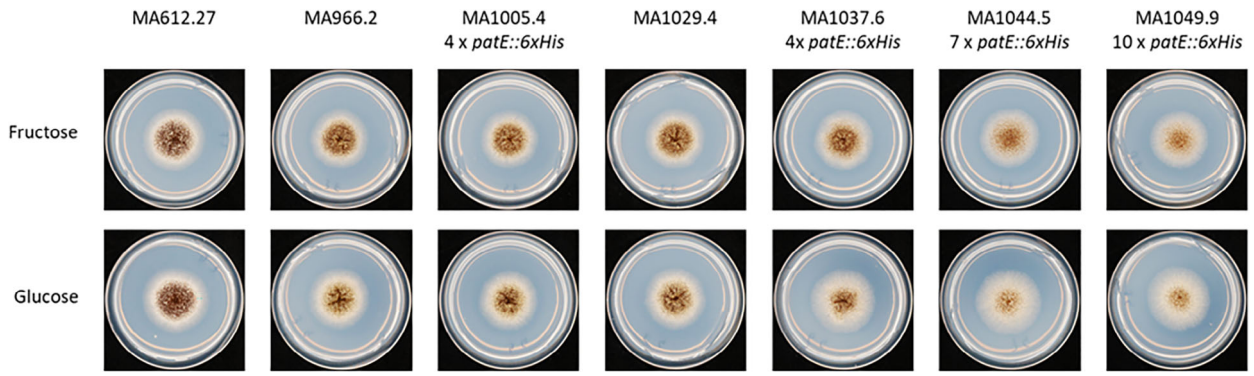



**Fig. 6.** Growth analysis of parental strains MA966.2 and MA1029.4 and *patE::His6* overexpressing strains (MA1005.4, MA1037.6, MA1044.5, and MA1049.9). Strains were grown for 4 days on minimal medium with 1% (w/v) carbon source as indicated before the picture of the colony was taken.

construction and cultured at 37 °C in Luria-Bertani medium, with ampicillin (100 $\mu g \cdot mL^{-1}$).

## CRISPR/Cas9 plasmid constructions

The gene deletion strains used in this study were created by using the marker-free CRISPR/Cas9 genome editing method [22] or by a one-step cloning of crRNAs into a newly constructed plasmid pLML001, derived from pFC332. sgRNA targets were designed using the CHOP-CHOP web-tool [35]. All primers used in this study are listed in Table S1.

The vector pLML001 supports a one-step sgRNA targeting sequence incorporation into the vector and utilizes an RNA polymerase III promoter for sgRNA transcription. Plasmid pLML001 was constructed as follows. The vector pFC332 used as backbone expresses an *A. niger* codon-pair-optimized *cas9* gene from *S. pyogenes*, a fungal *hph* gene for hygromycin resistance and a bacterial ampicillin resistance marker [20]. The vector carries an AMA1 sequence, which provides autonomous replication in many different fungal species. The pFC332 shuttle vector was linearized via digestion overnight at 37 °C using PacI, dephosphorylated and column purified. The transcription unit for the CRISPR sgRNA delivery was amplified from pFTK087 [36], using primers PacI_Pro_f and PacI_Pro_r (Table S1) to add *Pac*I restriction sites to the PCR product. The PCR fragment codes for the Pro1 tRNA promoter from *A. niger* together with its native ribozymes self-cleaved Pro1 tRNA sequence from *A. niger* [21], a *Esp*3I-removable *lacZα* expression fragment for blue/white selection, the tracrRNA sequence (guide RNA scaffold), and a *trpC* terminator sequence. The pFTK087 contains an improved tracrRNA sequence variant (single T to C substitution at the fifth nucleotide of the sgRNA scaffold). This variation is aiming at the disruption of a continuous sequence of thymines, which is the pause signal for RNA polymerase III and thus could potentially reduce transcription efficiency [37]. The amplified PCR product (1743 bp) was digested using *Pac*I overnight at 37 °C and inactivated for 20 min at 80 °C in a thermocycler and column purified. The PCR product was cloned into the linearized pFC332 vector DNA. Ligation mix was transformed to chemically competent *E. coli* DH5α cells via heat shock protocol, and cells were plated on LB containing 100 $\mu g \cdot mL^{-1}$ ampicillin, 40 $\mu g \cdot mL^{-1}$ XGAL, 0.1 mM IPTG. Plasmids from resulting blue colonies were isolated and the correctly assembled pLML001 vector was verified via Sanger sequencing. The assembled pLML001 vector contains two *Esp*3I restriction sites, flanking the *lacZα* expression fragment inside the sequence of the sgRNA transcription unit. After restriction with *Esp*3I, the created 4 bp-long sticky ends (GGCC and GTTT 5′–3′) are used to incorporate the ~ 20 bp-long targeting sequence of the sgRNA and creating a complete sgRNA transcription unit. Such inserts were constructed via annealed single-stranded DNA oligonucleotides, where the targeting sequences were ordered as complete complementary sequences for double-stranded DNA and the flanking single-stranded 4 bp-long sticky ends are used for the cloning (Table S1). Inserts were cloned into a *Esp*3I pre-digested pLML001 where the backbone is separated from the insert via gel extraction or incorporated via a one-pot restriction/ligation cloning. All plasmids used in this study are listed in Table S2.

## Donor DNA fragment constructions

To create donor DNA to delete target genes and at the same time integrate a GLS, we first constructed a DNA fragment consisting of the *PglaA* promoter region (−635 to −247 upstream of ATG) and the *Tgla* terminator region (+142 to +547 after stop codon). KORE sequences were introduced by ordering long primers including the KORE sequences at the 5′part of the primer used to amplify the *TglaA* fragment. These primers also have an overlap with the primers used to amplify the *PglaA* fragment, allowing fusion PCR to obtain

the entire PCR fragment (Table S1). The approach to obtain the gene construct to introduce landing sites is schematically depicted in Fig. S2. To generate the donor DNA fragment to delete the target gene and at the same time introduce the GLS, PCR primers were designed to amplify the 5′- and 3′-flanking regions of the target gene. The primers were designed to have overlaps to perform a three-fragment fusion PCR to create a DNA fragment that consists of the *PglaA-KORE-TglaA* fragment flanked by the 5′- and 3′-flanking regions of the target gene (Fig. S2). The fusion PCR product was directly used for transformation.

The KORE1 sites at the deleted *pepA*, *pepB* and *pepN* loci in MA966.2 were replaced by KORE4 sites (*pepA* and *pepB*) and KORE5 (*pepN*) by designing two new sgRNAs close to the 3′end of the left flanking region and close to the 5′end of the right flanking region of the respective gene. A new donor DNA with the new KORE sequence was used to repair the double strand breaks introduced at the respective loci (Fig. S3).

The *PglaA-patE::6xHis-TglaA* donor DNA was also generated by fusion PCR (Fig. S4). The final PCR fragment contains the *PglaA* promoter (634 bp), the *patE* gene containing a His-tag at its C-terminus and the *TglaA* terminator sequences (547 bp). The endogenous signal sequence for secretion of PatE protein was used and the His-tag was directly added to the C-terminal amino acid of PatE without any spacer amino acid. The 3232 bp PCR product was cloned into pJet2.1 to give pMA466 and sequenced to confirm that no PCR errors were introduced. The DNA fragment containing the *patE::6xHis* expression cassette was excised from pMA466 with *Pme*I, purified form agarose gel and used for transformation.

## Transformation of *A. niger* and analysis of the transformants

*Aspergillus niger* transformants were obtained by selection for hygromycin resistance as described [34] using a final concentration of 100 $\mu g \cdot mL^{-1}$ hygromycin. Deletion of genes was achieved by transforming strain MA612.27 (*Δku70*) with pFC332-sgRNA based or pLM001-sgRNA plasmid (both 2–5 μg DNA) and the repair DNA fragment (1–2 μg DNA). Transformants were purified on MM supplemented with hygromycin, followed by a purification step on MM to allow loss of the hygR-AMA1 plasmids. Afterwards, the loss of these plasmids was confirmed by growth analyses on MM supplemented with hygromycin and correct integration of the repair DNA was verified by diagnostic PCR.

## Cultivation of *A. niger* for protein analysis

To analyse the secretion of PatE::6xHis, transformants as well as the parental strains were cultured in shake flasks. To start the culture, freshly harvested spores ($1 \times 10^8$ spores) were inoculated in 300 mL Erlenmeyer flasks containing 100 mL of CM liquid medium and grown in a shaker at 200 r.p.m. and 30 °C for up to 4 days. After 24, 48, 72 and 96 h of growth, 5 mL of medium sample was taken, filtered through a sterile filter (Amplitude EcoCloth, delivered by VWR International), subsequently filter-sterilized (0.20 μm pore diameter) and small aliquots were directly frozen in liquid nitrogen and stored at −20 °C.

## SDS/PAGE and western blot analysis

Protein samples were thawed and to 12 μL of medium sample, 3 μL of 5× SDS sample buffer (0.5 M Tris–HCl pH 6.8, 10% SDS, 50% Glycerol, 0.75 M β-mercaptoethanol, 0.1% bromophenol blue) was added. Samples were boiled for 8 min at 100 °C. 10 μL of the samples (equivalent to 8 μL of medium sample) was loaded onto a precast SDS/PAGE gel (12% Mini-PROTEAN® TGX™ Precast Protein Gels, 4561046; Bio-Rad Laboratories, Ede, The Netherlands). Also, 5 μL of Spectra™ Multicolor Broad Range Protein Ladder (26634; Thermo Scientific, Breda, the Netherlands) was loaded onto the gel. Gels were run in a Bio-Rad Mini PROTEAN system, using 10× diluted TGS running buffer (1610772; Bio-Rad Laboratories). The gels were run for 45 min at 170 volts until the blue dye reached the bottom of the gel. The SDS/PAGE gels were stained overnight with SYPRO® Ruby Protein Gel Stain (1703125; Bio-Rad Laboratories) in the dark. After staining, the gels were de-stained with 10% MeOH and 7% acetic acid for 2 h. For imaging, a Gel Doc EZ Imaging System with IMAGE LAB software (Bio-Rad Laboratories) was used on normal settings for Sypro Ruby stained protein gels.

For western blot analysis, gels were run as described above and the SDS/PAGE gel was transferred to a Trans-Blot Turbo Transfer system (Bio-Rad Laboratories). The blotting sandwich was made using Trans-Blot Turbo Mini 0.2 μm Nitrocellulose Transfer Packs (1704158; Bio-Rad Laboratories). The transfer system was run with the 1 mini TGX gel pre-programmed protocol. After transferring the proteins from the SDS-gel to the nitrocellulose membrane, the membrane was washed with MQ water for 15 min, followed by a 15 min washing step in 1× TBS buffer (10× TBS: 24.2 g Tris pH 7.6, 80 g NaCl). Then, the membrane was blocked with a 5% milk solution in TBST (1 g Campina "ELK" skimmed milk powder in 20 mL TBST (1× TBS buffer + 0.05% Tween20)) for a minimum of 90 min. After blocking, the membrane was washed with MQ water. For detection of His-tagged proteins, anti-His antibody (6x-His Tag Monoclonal Antibody (3D5), HRP, R93125; Thermo Scientific) was 1 : 10 000 diluted in TBST and added to the membrane and incubated overnight. After antibody binding, the membrane was first washed with MQ for 15 min and then 4 times 5 min each with TBST. For detection of the HRP-conjugated antibody, TMB Enhanced One Component HRP Membrane Substrate (T9455; Sigma) was used.

### Protein determination

The supernatants of three biological replicates of parental or transformant strains grown in CM for 3 days were collected and stored at −20 °C as described above. These frozen supernatants were thawed and concentrated using Vivaspin® 500 columns (Vivaspin® 500, 10 000 MWCO, VS0102; Sartorius, Amersfoort, the Netherlands). 500 µL medium sample was loaded onto a column and centrifuged at 12 000 ***g*** for 5 min. The remaining volume (50 µL) was used to make a 4-fold dilution of the concentrated medium sample. Total protein concentration was determined in triplicate using the Quick Start™ Bradford Protein Assay Kit 1 (5000201; Bio-Rad Laboratories), according to the protocol supplied by the manufacturer.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

MA performed most of the experiments with the help of PKV, LM, TJGR-T and GT. The landing site approach was initially conceived by AFJR and JV and further fine-tuned together with MA and SJS. The biochemical work on PatE was done by GT and MWF. The paper was written by MA, JV and AFJR and edited by all authors. All authors read and approved the final manuscript.

## Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/febs.16891.

## Data availability statement

The Supplementary Tables and Figures are available at https://community.data.4tu.nl, doi: 10.4121/71c106ce-b416-4352-b6f2-511764b59302.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Schematic overview of the lineage of the PatE::His6 expressing strains and predecessor strains starting with wild-type strain N400.
**Fig. S2.** Schematic overview of the construction of donor DNA fragments for integration of the glucoamylase landing sites and simultaneous deletion of the target gene.
**Fig. S3.** Schematic overview of pepA locus, the DNA fragments, and primers used to change the KORE1 sequence in the glucoamylase landing site in pepA into the KORE4 sequence.
**Fig. S4.** Schematic overview of the construction of the PglaA-patE::6xHis-TglaA gene expression cassette.
**Table S1.** Primers used in this study.
**Table S2.** Plasmids used in this study.