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1 **CRISPR/Cas9 facilitates rapid generation of constitutive forms of transcription factors in**
2 ***Aspergillus niger* through specific on-site genomic mutations resulting in increased**
3 **saccharification of plant biomass**

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11

12 **Abstract**

13 The CRISPR/Cas9 system has been successfully applied for gene editing in filamentous fungi.
14 Previous studies reported that single stranded oligonucleotides can be used as repair templates
15 to induce point mutations in some filamentous fungi belonging to genus *Aspergillus*. In *Aspergillus*
16 *niger*, extensive research has been performed on regulation of plant biomass degradation,
17 addressing transcription factors such as XlnR or GaaR, involved in (hemi-)cellulose and pectin
18 utilization, respectively. Single nucleotide mutations leading to constitutively active forms of XlnR
19 and GaaR have been previously reported. However, the mutations were performed by the
20 introduction of versions obtained through site-directed or UV-mutagenesis into the genome. Here
21 we report a more time- and cost-efficient approach to obtaining constitutively active versions by
22 application of the CRISPR/Cas9 system to generate the desired mutation on-site in the *A. niger*
23 genome. This was also achieved using only 60-mer single stranded oligonucleotides, shorter than
24 the previously reported 90-mer strands. In this study, we show that CRISPR/Cas9 can also be
25 used to efficiently change functional properties of the proteins encoded by the target gene by on-
26 site genomic mutations in *A. niger*. The obtained strains with constitutively active XlnR and GaaR
27 versions resulted in increased production of plant biomass degrading enzymes and improved
28 release of D-xylose and L-arabinose from wheat bran, and D-galacturonic acid from sugar beet
29 pulp.

30 **Keywords:** CRISPR/Cas9; filamentous fungi; constitutive transcription factor; CAZyme

31

1. Introduction

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system originates from bacterial and archaeal immune systems. Cas proteins play a role in foreign sequence acquisitions, as well as disruption of exogenous DNA through endonuclease activity of some of these proteins, such as Cas9 [1]. In the CRISPR/Cas9 system, Cas9 forms a complex with a CRISPR RNA (crRNA) [2], which originates from exogenous protospacer sequences, and a trans-activating CRISPR RNA (tracrRNA) [3]. Due to crRNA-protospacer homology, the Cas9-crRNA-tracrRNA complex will be directed to the target locus, where the Cas9 endonuclease interacts with the target DNA strand through a protospacer adjacent motif (PAM), unwinds the DNA strand, and performs a double-strand break three nucleotides upstream of the PAM [4]. This system was adapted for genetic engineering using designed synthetic single-guide RNAs instead of the original crRNA-tracrRNA complex [5] and it has been successfully applied in a variety of eukaryotic organisms [6-8], including efficiently plant biomass degrading filamentous fungi [9]. However, its application has mainly focused on the inactivation of genes through deletions, point mutations or on the insertion of genes at specific loci [9-12].

Plant biomass is the most abundant carbon source on earth and it consists mainly of plant cell wall polysaccharides (cellulose, hemicelluloses and pectin), and the aromatic polymer lignin. These polymers form a complex network, ensuring the strength and rigidity of plant cells [13]. The complex structure of plant biomass requires a broad set of hydrolytic and oxidative enzymes to degrade it. Filamentous fungi are efficient plant biomass degraders due to their ability to produce and secrete large amounts of Carbohydrate Active enZymes (CAZymes, www.cazy.org [14]). Fungal enzymes also have large variety of applications in many industrial fields such as food and feed, pulp and paper or textile and detergent industries [15].

The production of enzymes required for plant biomass degradation is regulated by transcription factors, which can act as transcriptional activators or repressors [16]. Many transcription factors have been described in ascomycetous fungal model organisms such as *Neurospora crassa*, and in organisms involved in industrial applications such as *Aspergillus niger, Aspergillus oryzae* and *Trichoderma reesei* [17].

The xylanolytic transcription factor XlnR from *A. niger* was the first described fungal regulator involved in (hemi-)cellulose utilization [18]. It was also shown that a single V756F point mutation in the C-terminal region of the *xlnR* gene results in a fully active transcription factor, even under repressing conditions [19]. Hasper et al. suggested that mutations in the C-terminal region of XlnR

64 disturb a putative inhibitory domain, which would normally turn this transcription factor into an
65 inactive form [19]. A similar A871V point mutation in the C-terminal region of the *Penicillium*
66 *oxalicum* XlnR ortholog also resulted in enhanced expression of lignocellulolytic enzymes [20].

67 The pectinolytic transcription factor GaaR was also reported to show constitutive activity caused
68 by a single point mutation in *A. niger* [21]. The endogenous *gaaR* gene was deleted and replaced
69 with a DNA construct carrying a W361R point mutation. Alazi et al. [21] proposed that this mutation
70 disrupts the interaction between GaaR and its repressor, GaaX [22], under non-inducing
71 conditions.

72 So far, attempts to generate constitutively active transcription factor mutants involved either in
73 site-directed mutagenesis of the target gene and its insertion in a specific genomic loci [19], the
74 deletion of entire C-terminal regions of the target genes [23], or the insertion of a mutant allele in
75 the deleted locus of the endogenous gene [21]. These are relatively labor-intensive approaches,
76 which also may cause subtle additional changes at the site of integration or deletion that could
77 further effect the phenotype. To demonstrate the versatility of CRISPR/Cas9-mediated genome
78 editing, beyond the generation of loss-of-function deletions or point mutations and gene
79 insertions, in this study we applied it to the generation of specific point mutations on site in the
80 native genomic copy of *xlnR* and *gaaR*, resulting in the previously reported constitutively active
81 versions of the regulators. The exoproteomes of the mutant strains were evaluated by SDS-PAGE
82 and enzyme activity analyses, and their ability to saccharify crude plant biomass substrates was
83 assessed, to confirm the functionality of the mutated versions of the regulators.

84 ## 2. Material and methods

85 *2.1 Strains, media and growth conditions*

86 *Escherichia coli* DH5α was used for plasmid propagation, and was grown in Luria-Bertani (LB)
87 medium supplemented with 50 µg mL^{-1} ampicillin (Sigma Aldrich). Fungal strains used in this
88 study were derived from the *A. niger* CBS138852 strain. The generated mutants were deposited
89 at the culture collection of Westerdijk Fungal Biodiversity Institute under accession numbers
90 indicated in Table 1.

91 Table 1. *A. niger* strains used in this study.

CBS number	Strain description	Genotype	Point mutations	Reference
CBS 138852	N593 Δ*kusA*	*cspA1*, *pyrG^{-}*, *kusA::amdS*		[24]
CBS 145907	XlnR V756F	*cspA1*, *pyrG^{-}*, *kusA::amdS*	G2330T	This study
CBS 145908	GaaR W361R	*cspA1*, *pyrG^{-}*, *kusA::amdS*	T1285C, C1293T	This study

92

93 Strains were grown at 30˚C in Aspergillus Minimal Medium (MM) or Complete Medium (CM) [25]
94 supplemented with 1% D-glucose and 1.22 gL^{-1} uridine (Sigma Aldrich).

95 For liquid cultures, freshly harvested spores were pre-grown in CM containing 2% D-fructose and
96 1.22 gL^{-1} uridine for 16 h at 30˚C in a rotary shaker at 250 rpm. The mycelium was harvested by
97 filtration through sterile cheesecloth, rinsed with MM, and approximately 2.5 g (wet weight)
98 mycelium was transferred into 50 mL MM containing 0.45% D-fructose (corresponding to 25 mM)
99 or 2% D-fructose, 1% wheat bran (WB) or 1% sugar beet pulp (SBP). Supernatant samples were
100 taken after 24 h incubation at 30˚C in a rotary shaker at 250 rpm. The samples were centrifuged
101 (20 min, 3220 *× g,* 4˚C) and cell-free supernatant samples were stored at -20˚C until further
102 processing.

103 *2.2 Construction of mutant strains*

104 The ANEp8-Cas9-*pyrG* plasmid, which contains the autonomous fungal replicating element
105 AMA1,*pyrG* as selection marker, *cas9* gene and the guide RNA (gRNA) expression construct
106 under the control of the proline transfer ribonucleic acid ($tRNA^{Pro1}$) promoter, was used in this
107 study [26]. The ANEp8-Cas9-*pyrG* plasmids (Fig. S1) were constructed according to the protocol
108 described by Song et al. [26]. The gRNA sequences were predicted using Geneious 11.1.4
109 software (https://www.geneious.com), and P1-P4 primers (Table S1) were used for the
110 amplification of the gRNA expression constructs, which were cloned into the ANEp8-Cas9-*pyrG*

111 plasmids and subsequently transformed into *E. coli*. Correct clones were identified by PCR
112 amplification of the gRNA coding region by using the Fw-screen and Rev-screen primers (Table
113 S1). All primers used in this study were ordered from Integrated DNA Technologies, Inc. (IDT,
114 Leuven, Belgium).

115 Single-stranded DNA 60-mer, 90-mer or 200-mer oligonucleotides carrying specific point
116 mutations (Table S2) (IDT, Leuven, Belgium) were designed to be used as repair templates to
117 repair the double stranded DNA breaks caused by Cas9. Multiple templates were used for the
118 introduction of GaaR W361R mutation, including templates with extended length or multiple point
119 mutations flanking the target site to facilitate a successful T → C transition in the nucleotide
120 position 1285.

121 *A. niger* protoplasting and transformation were performed as described by Kowalczyk et al. [27],
122 with minor modifications. One µg ANEp8-Cas9-*pyrG* plasmid, together with 5 µg of each
123 corresponding repair template were used for each transformation. Putative mutant strains were
124 purified by two consecutive single colony streaking, followed by cultivation on uridine-containing
125 plates in order to remove the self-replicating AMA1 plasmid [28]. Candidates carrying the
126 expected mutations were subsequently grown on medium containing 5-fluoro-orotic acid (5-FOA)
127 in order to screen for colonies, which have lost the ANEp8-Cas9-*pyrG* plasmid. All *A. niger*
128 mutants were confirmed by Sanger sequencing (Macrogen Europe, Amsterdam, the Netherlands)
129 (Fig. S2) using the sequencing primers listed in Table S1.

130

131 *2.3 SDS-PAGE and enzyme activity assays*

132 Culture filtrates of the control and mutant strains grown in media containing WB or SBP for 24 h
133 were used to evaluate the produced extracellular CAZymes and their activities.

134 Twelve µL of the culture filtrates was added to 4 µL loading buffer (10% of 1 M Tris–HCl, pH 6.8;
135 42% Glycerol, 4% (w/v) SDS; 0.02% (w/v) bromophenol blue; 4% of 14.7 M mercaptoethanol),
136 incubated at 85˚C for 15 min, ice-cooled for 2 min and centrifuged at ~ 10,000 *x g* for 2 min.
137 Finally, 10 µL were loaded onto 12% (w/v) acrylamide SDS-PAGE gels calibrated with PageRuler
138 Plus prestained protein marker (Thermo Scientific), and silver stained [29] and documented using
139 HP Scanjet G2410 scanner. All samples were evaluated in biological duplicates.

140 Enzyme activities were evaluated based on colorimetric para-nitrophenol (pNP) assays. Ten µL
141 supernatant samples were mixed with 10 µL 0.1% 4-nitrophenyl β-D-xylopyranoside (for β-

142 xylosidase activity), 0.1% 4-nitrophenyl β-D-galactopyranoside (for β-1,4-D-galactosidase
143 activity) or 0.1% 4-nitrophenyl α-L-arabinofuranoside (for α-L-arabinofuranosidase activity)
144 substrates, 50 µL 50 mM NaAc (pH 5) and 30 µL demineralized water in a final volume of 100 µL.
145 β-xylosidase and β-1,4-D-galactosidase activities were measured after 1 h incubation at 30˚C,
146 while the α-L-arabinofuranosidase activity was measured after 30 min incubation at 30˚C. The
147 reactions were stopped by the addition of 100 µL of 0.25 M Na_2CO_3 and absorption values were
148 measured at 405 nm wavelength using FLUOstar OPTIMA (BMG Labtech). All measurements
149 were performed by using both technical and biological triplicates. Differences in enzyme activities
150 were determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

151

152 *2.4 Saccharification tests*

153 Saccharification reactions were performed in 96-well flat bottom microtiter plates. Each reaction
154 contained 20 µL culture filtrate mixed with 50 mM sodium citrate (pH 5) containing 3% WB or 3%
155 SBP in a final volume of 250 µL. The reactions were incubated for 6 h at 30˚C and 400 rpm.
156 Reactions were stopped by heat inactivation of enzymes for 15 min at 95˚C. Plates were
157 centrifuged for 20 min at 3220 *× g*, and the supernatants were 10-fold diluted in MilliQ water prior
158 to analysis. The experiment was performed using biological and technical triplicates.

159 Monosaccharides were analyzed from peak areas in HPAEC-PAD (Dionex ICS-5000 + system;
160 Thermo Scientific) equipped with CarboPac PA1 column (2×250 mm with 2×50 mm guard column;
161 Thermo Scientific). The column was pre-equilibrated with 18 mM NaOH followed by a multi-step
162 gradient: 0-20 min: 18 mM NaOH, 20-30 min: 0-40 mM NaOH and 0-400 mM sodium acetate, 30-
163 35 min: 40-100 mM NaOH and 400 mM to 1 M sodium acetate, 35-40 min: 100 mM NaOH and 1
164 M to 0 M sodium acetate followed by re-equilibration of 18 mM NaOH for 10 min (20°C; flow rate:
165 0.30 mL/min). Between 5-250 mM D-glucose, D-xylose, L-arabinose and D-galacturonic acid
166 (Sigma-Aldrich) were used as standards for quantification. Blank samples containing 3% WB or
167 SBP, without the addition of culture filtrates were measured and the values were subtracted from
168 each corresponding saccharification test result in order to exclude the amount of free sugar
169 already present in the experimental condition. Differences in saccharification efficiency were
170 determined using Student's two-tailed type II *t*-test. Significance was regarded as $p < 0.05$.

3. Results and discussion

3.1 CRISPR/Cas9 facilitates efficient on-site functional mutations

In order to achieve precise point mutations without unspecific genomic alterations, such as random insertions or deletions, we used *A. niger* Δ*kusA* as receptor strain for all our transformations [24]. Due to the lack of non-homologous end joining (NHEJ) DNA repair pathway caused by the *kusA* deletion, Cas9 double strand breaks must be repaired with a repair template homologous to the target DNA region, facilitating the implementation of short templates carrying specific point mutations. The repaired DNA strand may still serve as a target region for further Cas9 cutting events, so due to the lack of NHEJ, it is important to introduce intended alterations of the protospacer or PAM sequence in order to avoid further double strand DNA breaks, leading to the death of the mutant colonies.

Nødvig et al. previously described that 90-mer single stranded oligonucleotides could be used for successful introduction of nonsense codons into the pigmentation gene *yA*, *alba* and *wA* of *A. nidulans, A. oryzae* and *A. niger*, respectively. It was also shown that the DNA repair did not show any preference for the targeted sense or anti-sense strand [30]. Based on this, we decided to use repair templates complementary to the anti-sense strand of the target DNA.

First, we performed a single GTC → TTC nucleotide mutation in the *xlnR* coding region, resulting in a valine-756-phenylalanine (V756F) mutation [19]. The gRNA sequence (P3-XlnR, P4-XlnR, Table S1) closest to the nucleotide of interest was predicted by Geneious. The 90-mer oligonucleotide repair template (XlnR repair template, Table S2) did not require any additional point mutations, since the target codon was also part of the PAM sequence, ensuring that the Cas9 endonuclease would not be able to re-bind and cut the repaired sequence anymore. After fungal transformation, three randomly chosen candidates were sequenced in their CRISPR/Cas9 target site (Fig. 1A). All three candidates were shown to be correct and candidate 3 was randomly selected for further phenotype evaluation.

To obtain a constitutively active GaaR [21], a T → C transition in a TGG codon is required, resulting in a tryptophan-361-arginine (W361R) mutation. The gRNA sequence (P3-GaaR, P4-GaaR, Table S1) with the highest on-target activity was predicted by Geneious based on the experimentally determined predictive model proposed by Doench, et al. [31]. Contrary to the *xlnR* point mutation design, an additional mutation was required in order to avoid re-cutting of the repaired target strand by the CRISPR/Cas9 system. Previous studies reported that the

202 CRISPR/Cas9 system shows tolerance to mismatches in the protospacer sequence [32], which
203 led us to alter the PAM sequence with a G → C silent mutation.

204 A 90-mer single stranded oligonucleotide was designed to introduce the intended point mutations,
205 interspaced by 52 nucleotides (GaaR repair template 1, Table S2). After transformation, four
206 randomly selected candidates were submitted for sequencing. Sequencing results (Fig. 1B)
207 showed the intended alteration of the PAM sequence, although the W361R mutation did not take
208 place. The same transformation was attempted with a longer 200-mer oligonucleotide repair
209 template (GaaR repair template 2, Table S2). All sequenced colonies carried only the PAM
210 sequence altering mutation (data not shown). Both attempts suggest that the repair templates
211 were not entirely incorporated, excluding the W361R mutation, most likely due to the large
212 distance between the two mutated nucleotides.

213 Taking into account these results, a re-designed gRNA encoding sequence (P3.2-GaaR, P4.2-
214 GaaR, Table S1) closer to the target nucleotide was performed. Similarly to the previous
215 approach, a new repair template was designed carrying two point mutations (2 PM), but this time
216 the mutations were interspaced by only seven nucleotides. Since the new repair template carried
217 both the intended W361R and the PAM sequence mutations closer to each other, we decided to
218 reduce the length of the repair template to 60 oligonucleotides, which would theoretically induce
219 homologous recombination (GaaR repair template 2 PM, Table S2). In addition, another repair
220 template was designed carrying five additional silent mutations (GaaR repair template 7 PM,
221 Table S2) in order to hinder the homology of the sequence around the nucleotide of interest, to
222 avoid the previously observed results where only the PAM sequence alteration occurred. Five
223 transformant colonies were sequenced for each transformation, resulting in four correct mutants
224 each (Fig. 1C). Interestingly, the 2 PM repair template resulted in one colony carrying only the
225 PAM sequence altering mutation, where most likely a recombination happened with ≤ 12
226 nucleotides serving as 5'-end flanking region, suggesting that even shorter repair templates could
227 successfully restore the damaged DNA. This would be especially relevant when CRISPR/Cas9
228 genome editing is performed in an *A. niger* strain of a different lineage, whose genomic DNA
229 sequence is likely not fully identical. The colony 2 PM 1 was selected for further phenotypic
230 evaluation.

231

232 *3.2 Constitutive versions of XlnR and GaaR result in elevated enzyme levels*

233 Two crude plant biomass substrates were chosen for phenotypic characterization of XlnR V756F
234 and GaaR W361R mutants. WB is rich in glucuronoarabinoxylan, suitable for characterization of
235 a constitutive XlnR phenotype, whereas SBP has a high pectin content and was previously used
236 for the characterization of *gaaR* deletion mutants [27, 33].

237 SDS-PAGE and enzyme activity assays of 24 h culture filtrates after growth of the mutants and
238 control strain on 1% WB and 1% SBP were assessed for phenotypic characterization. SDS-PAGE
239 results of the XlnR V756F mutant grown in 2% D-fructose, a carbon source showing low carbon
240 catabolite repression (CCR) mediated gene repression [34], showed the presence of mainly
241 putative endoxylanases (13-33 kDa) and β-xylosidases (122 kDa) [13] (Fig. 2A), which were not
242 present in the control strain, demonstrating the inducer-independent constitutive action of XlnR.
243 The xylanolytic enzymes were more abundant when the mutant was grown in medium containing
244 0.45% D-fructose (Fig. 2B), most likely due to the reduced CCR effect mediated by CreA
245 compared to the 2% D-fructose culture [35, 36]. Cultivation of the XlnR V756F mutant in 1% WB
246 medium also resulted in an increase of the major putative xylanolytic enzymes compared to the
247 control strain (Fig. 2C), suggesting improved saccharification abilities in this mutant.

248 The cultivation of the GaaR W361R mutant in liquid medium containing 2% D-fructose as a sole
249 carbon source did not result in an increased production of pectinolytic enzymes as the SDS-PAGE
250 pattern was identical to that of the control strain (data not shown). However, the samples from 1%
251 SBP cultures showed elevated levels of CAZymes, especially in the 35-66 kDa range (Fig. 2D),
252 where most *A. niger* endo- and exopolygalacturonases, and pectin lyases are found [13]. The
253 genes encoding these enzymes have been shown to be controlled by GaaR [37].

254 The SDS-PAGE profiles were confirmed by enzyme activity assays. β-xylosidase activity (BXL)
255 was 53% increased in the XlnR V756F supernatant from 1% WB cultures compared to its control
256 strain (Fig. 3A). Moreover, BXL activity in the culture filtrate of this mutant showed very similar
257 values when cultivated in 1% SBP, while the control strain did not show any BXL activity under
258 this condition, due to the lack of activation of XlnR (Fig. 3B). This result also proves that the XlnR
259 V756F mutant can express its target genes in non-inducing conditions. Interestingly, the
260 supernatant from GaaR W361R mutant showed a 31% increase in β-1,4-D-galactosidase activity
261 (LAC) in WB medium. However, LAC activity was 63% reduced in samples of the SBP cultures,
262 compared to the control. Since the regulation of β-galactosidase genes has been shown to be
263 controlled by a broad range of transcription factors, including GaaR [37], the increase of LAC
264 activity in WB medium and decrease in SBP medium may not be related to a direct constitutive
265 GaaR effect, but rather to an altered interaction between the transcription factors controlling the

266 production of this activity. Finally, the XlnR V756F mutant showed 15% and 10% reduced α-L-
267 arabinofuranosidase activity (ABF) in the WB and SBP samples, respectively. In contrast, the
268 GaaR W361R mutant showed a 22% and 6% increase in ABF activity in WB and SBP cultures,
269 respectively, suggesting that the constitutive GaaR rather has an (minor) activating role in the
270 expression of *abf* genes. Overall, the low fold change values suggest that neither XlnR V756F,
271 nor GaaR W361R play an essential role in the activation of these genes, most likely because they
272 are mainly controlled by the arabinanolytic regulator AraR [37].

273

274 *3.3 The enzyme mixtures from the constitutive regulator strains resulted in improved*
275 *saccharification of wheat bran and sugar beet pulp*

276 Saccharification tests were performed using the 24 h culture filtrates of XlnR V756F, GaaR
277 W361R and the control strain cultured on 1% WB or 1% SBP (Fig. 4) (subsequently referred to
278 as WB culture filtrate and SBP culture filtrate, respectively). Both crude substrates were used in
279 order to test the phenotype of each mutant strain under inducing and non-inducing conditions.

280 The release of D-xylose, L-arabinose, D-galacturonic acid and D-glucose from 3% WB and 3%
281 SBP by the WB and SBP culture filtrates was measured. D-xylose release from xylan is regulated
282 by XlnR, while the release of D-xylose from pectin has been suggested to be co-regulated by
283 XlnR and GaaR [27, 37]. The release of L-arabinose is mainly controlled by the arabinanolytic
284 transcription factor, AraR [38], but XlnR and GaaR have also been reported to co-regulate some
285 arabinanolytic genes [27, 37]. The release of D-galacturonic acid from pectin is regulated by GaaR
286 [33]. Finally, D-glucose can either be released from cellulose or starch. SBP is rich in cellulose,
287 while WB contains both cellulose and starch [39]. Since neither XlnR nor GaaR was shown to
288 play a role in starch utilization, D-glucose release would be most likely related only to cellulose
289 utilization, in which XlnR is involved, as first suggested by van Peij et al. [40].

290 Saccharification of WB (Fig. 4) showed increased release of D-xylose (Fig. 4A) and L-arabinose
291 (Fig. 4B) by the XlnR V756F mutant for both WB and SBP culture filtrates. When the SBP culture
292 filtrates were used, D-xylose release by XlnR V756F was especially significant compared to the
293 control strain, which did not release D-xylose due to the lack of XlnR induction (Fig. 4A). The
294 GaaR W361R mutant showed a similar amount of released D-xylose (Fig. 4A) compared to the
295 control for both culture filtrates, while L-arabinose release (Fig. 4B) was similar for the WB culture
296 filtrate, but reduced for the SBP culture filtrate. This could be associated with a competing effect

297 between GaaR and XlnR [27], also supported by the fact that the constitutive XlnR resulted in a
298 significant increase of L-arabinose release in this condition.

299 D-galacturonic acid is present in very low amounts in WB, most likely found only in thin layers of
300 pectin located under the outer and the epidermal cuticles [39, 41]. Our results show that SBP
301 culture filtrate from XlnR V756F generated the highest D-galacturonic acid release (Fig. 4C),
302 probably due to the more abundant presence of β-xylosidases catalyzing the removal of D-xylose,
303 which can decorate pectin. This could facilitate the degradation of the galacturonan backbone by
304 the pectinolytic enzymes also present in the SBP culture filtrates. Regarding D-glucose release
305 (Fig. 4D), the XlnR V756F mutant showed a similar value compared to the control strain when
306 WB culture filtrate was used. However, for the SBP culture filtrate, the XlnR V756F mutant showed
307 significantly higher D-glucose release, which is most likely related to the improved xylanolytic
308 activities, making cellulose more accessible for degradation in WB. Both GaaR W361R culture
309 filtrates showed reduced D-glucose release from WB, possibly due to an antagonistic effect
310 between GaaR and XlnR [27], resulting in decreased xylanolytic activity on hemicellulose in the
311 constitutive GaaR mutant, thus reducing cellulose degradation.

312 In case of SBP saccharification (Fig. 5), all strains released similar levels of D-xylose when WB
313 culture filtrates were used (Fig. 5A). In contrast, the XlnR V756F SBP culture filtrate showed a
314 significant improvement in D-xylose release compared to the control. Regarding L-arabinose
315 release (Fig. 5B), SBP culture filtrates showed higher saccharification efficiency compared to that
316 of WB culture filtrates. However, the mutants did not show improved L-arabinose release
317 compared to the control strain, which indicates that other transcription factors, such as AraR, have
318 a more predominant role in releasing L-arabinose from pectin. These results also suggest that
319 the XlnR V756F and/or GaaR W361R mutants has a negative effect over AraR-mediated L-
320 arabinose release from this substrate. In contrast, D-galacturonic acid release was significantly
321 improved by both GaaR W361R culture filtrates (Fig. 5C), especially in the case of WB culture
322 filtrates, where the control strain released only a minimal amount of D-galacturonic acid due to
323 the lack of GaaR induction. Finally, D-glucose release was similar for the WB culture filtrate
324 samples, while both mutants showed a slight decrease compared to the control when SBP culture
325 filtrates were used (Fig. 5D).This suggests that neither XlnR nor GaaR have a major influence on
326 cellulose degradation under these conditions.

327 Overall, the release of D-xylose and L-arabinose from WB was improved by the XlnR V756F
328 mutant. D-xylose release from both WB and SBP using the non-inducing condition for XlnR (SBP
329 culture filtrate) highlights the constitutive activity of the XlnR V756F mutant. D-galacturonic acid

release from SBP was improved by the GaaR W361R mutant, highlighting the constitutive activity in case of the non-inducing WB culture filtrates. The results of D-glucose release from SBP suggests that the cellulolytic activities of each sample are comparable, which justifies the results observed in case of WB saccharification, in which xylan degradation is the bottleneck of cellulose utilization.

4. Conclusions

In this work, we demonstrate how CRISPR/Cas9 genome editing can be used to efficiently modify the functionality of transcriptional regulators in *A. niger* by generating on-site mutation in the native copy of the corresponding genes in the genome. This also indicates that similar strategies could be used to change enzyme properties by mutating enzyme encoding genes, as well as many other functional mutations, further expanding the versatility of this genome editing approach. We used single stranded 60-mer or 90-mer oligonucleotide-mediated gene editing to generate constitutively active XlnR and GaaR transcription factors, but our data suggests that even shorter fragments could be used as templates to repair the Cas9-induced DNA strand cuts. The XlnR V756F mutant secreted a higher amount of CAZymes involved in the release of D-xylose and L-arabinose from WB confirming the functional mutation. Moreover, D-glucose release was also improved, likely facilitated by degradation of xylan, making cellulose more accessible for degradation in non-inducing conditions. Finally, the GaaR W361R mutant showed enhanced release of D-galacturonic acid from SBP. Overall, the use of CRISPR/Cas9 to generate such overproduction strains significantly reduced time and efforts compared to traditional approaches.

5. Conflict of interests

The authors declare that they have no competing interests.

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463

464 ## 8. Footnotes

465 Figure 1. Sanger sequencing results of the XlnR V756F and GaaR W361R mutant candidates. A)
466 Sequencing results of mutant candidates transformed with XlnR repair template. All three candidates
467 showed the expected mutation. Sequences show the whole coverage of the used repair templates.
468 Reference sequence is highlighted in black. The location of target nucleotides are highlighted in gray, while
469 the introduced mutations are indicated in red. The PAM sequence is shown in green. The protospacer
470 sequence is highlighted in yellow and the Cas9 cutting site is represented by a red bar. B) Sequencing
471 results of mutant candidates transformed with GaaR repair template 1 (Table S2). All four sequenced
472 candidates showed the introduction of the PAM sequence altering mutation. However, the W361R mutation
473 did not occur. C) Sequencing results of mutant candidates transformed with GaaR repair template 2 point
474 mutations (PM) or 7 PM (Table S2). Four candidates transformed with the 2 PM or 7 PM template showed
475 the expected mutations. The 2 PM candidate 5 showed only the introduction of the PAM sequence altering
476 mutation, while 7 PM candidate 5 showed a target sequence identical to the reference sequence. Results
477 of (B) and (C) are aligned in order to emphasize the new guide RNA selection for the transformations using
478 the 2 PM or 7 PM repair templates. Color codes as in (A).

479 Figure 2. Enzyme production analysis of XlnR V756F and GaaR W361R mutant strains. SDS-PAGE
480 analysis of the supernatants of *A. niger* CBS138852 (control strain), XlnR V756F and GaaR W361R
481 mutants after 24 h incubation in different culture conditions as indicated from A-D.

482

483 Figure 3. Enzyme activity of the supernatants from XlnR V756F and GaaR W361R mutant strains and from
484 *A. niger* CBS138852. The 24 h culture filtrates originated from 1% wheat bran (WB) (A) or 1% sugar beet
485 pulp (SBP) (B). Different enzyme activities are indicated by gray scale color codes. The values represent
486 the mean and standard deviation of the amount of released pNP measured at 405 nm wavelength.
487 Experiments were carried out using biological and technical triplicates. Bxl = β-xylosidase, Lac = β-1,4-D-
488 galactosidase, Abf = α-L-arabinofuranosidase. Statistical significance is represented by (*) ($p < 0.05$).

489

490 Figure 4. Monosaccharides released from 3% wheat bran (WB) by culture filtrates from control strain *A.*
491 *niger* CBS138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (A), L-
492 arabinose (B), D-galacturonic acid (C) and D-glucose (D) released after 6 h incubations with WB or sugar
493 beet pulp (SBP) culture filtrates are indicated by black and gray bars, respectively. Values represent the
494 mean and standard deviation of sugar concentration indicated in millimoles (mM). Experiments were carried
495 out using biological and technical triplicates. Statistical significance is represented by (*) ($p < 0.05$).

496

497 Figure 5. Monosaccharides released from 3% sugar beet pulp (SBP) by culture filtrates from control strain
498 *A. niger* CBS138852, and XlnR V756F and GaaR W361R mutant strains. The amount of D-xylose (A), L-
499 arabinose (B), D-galacturonic acid (C) and D-glucose (D) released after 6 h incubations with wheat bran
500 (WB) or SBP culture filtrates are indicated by black and gray bars, respectively. Values represent the mean
501 and standard deviation of sugar concentration indicated in millimoles (mM). Experiments were carried out
502 using biological and technical triplicates. Statistical significance is represented by (*) ($p < 0.05$).