Maltotriose consumption by hybrid *Saccharomyces pastorianus* is heterotic and results from regulatory cross-talk between parental sub-genomes

Heterotic origin of maltotriose consumption in Saccharomyces pastorianus

Nick Brouwers[a] , Anja Brickwedde[a], Arthur R. Gorter de Vries[a], Marcel van den Broek[a], Susan M. Weening[a], Lieke van den Eijnden[a], Jasper A. Diderich[a], Feng-Yan Bai[b], Jack T. Pronk[a] and Jean-Marc G. Daran[a]*

[a]Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
[b]State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China.
* Corresponding author: Jean-Marc G. Daran, j.g.daran@tudelft.nl, Department of Biotechnology, Delft University of Technology, van der Maasweg 9, 2629 HZ Delft, The Netherlands

Nick Brouwers:	N.Brouwers-1@tudelft.nl	
Anja Brickwedde:	a.brickwedde@web.de	
Arthur R. Gorter de Vries:	A.R.GorterdeVries@tudelft.nl	0000-0002-0841-6583
Marcel van den Broek:	Marcel.vandenBroek@tudelft.nl	
Susan M. Weening:	S.M.Weening@tudelft.nl	
Lieke van den Eijnden:	A.F.vandenEijnden@student.tudelft.nl	
Jasper A. Diderich:	J.A.Diderich@tudelft.nl	
Feng-Yan Bai:	baify@im.ac.cn	
Jack T. Pronk:	J.T.Pronk@tudelft.nl	0000-0002-5617-4611
Jean-Marc G. Daran:	J.G.Daran@tudelft.nl	0000-0003-3136-8193

Keywords: *S. eubayanus*, α-oligo-glucoside metabolism, heterosis, domestication, experimental evolution sequencing, brewing, hybridization.

Abstract

S. pastorianus strains are hybrids of *S. cerevisiae* and *S. eubayanus* that have been domesticated for several centuries in lager-beer brewing environments. As sequences and structures of *S. pastorianus* genomes are being resolved, molecular mechanisms and evolutionary origin of several industrially relevant phenotypes remain unknown. This study investigates how maltotriose metabolism, a key feature in brewing, may have arisen in early *S. eubayanus* x *S. cerevisiae* hybrids. To address this question, we generated a near-complete genome assembly of Himalayan *S. eubayanus* strains of the Holarctic subclade. This group of strains have been proposed to be the origin of the *S. eubayanus* subgenome of current *S. pastorianus* strains. The Himalayan *S. eubayanus* genomes harbored several copies of a *SeAGT1* α-oligoglucoside transporter gene with high sequence identity to genes encountered in *S. pastorianus*. Although Himalayan *S. eubayanus* strains are unable to grown on maltose and maltotriose, their maltose-hydrolase and *SeMALT1* and *SeAGT1* maltose-transporter genes complemented the corresponding null mutants of *S. cerevisiae*. Expression, in a Himalayan *S. eubayanus* strain, of a functional *S. cerevisiae* maltose-metabolism regulator gene (*MALx3*) enabled growth on oligoglucosides. The hypothesis that the maltotriose-positive phenotype in *S. pastorianus* is a result of heterosis was experimentally tested by constructing a *S. cerevisiae* x *S. eubayanus* laboratory hybrid with a complement of maltose-metabolism genes that resembles that of current *S. pastorianus* strains. The ability of this hybrid to consume maltotriose in brewer's wort demonstrated regulatory cross talk between sub-genomes and thereby validated this hypothesis. These results provide experimental evidence of the evolutionary origin of an essential phenotype of lager-brewing strains and valuable knowledge for industrial exploitation of laboratory-made *S. pastorianus*-like hybrids.

Importance

S.pastorianus, a *S.cerevisiae* X *S.eubayanus* hybrid, is used for production of lager beer, the most produced alcoholic beverage worldwide It emerged by spontaneous hybridization and have colonized

54 early lager-brewing processes. Despite accumulation and analysis of genome sequencing data of
55 *S.pastorianus* parental genomes, the genetic blueprint of industrially relevant phenotypes remains
56 unknown. Assimilation of wort abundant sugar maltotriose has been postulated to be inherited from
57 *S.cerevisiae* parent. Here, we demonstrate that although Asian *S.eubayanus* isolates harbor a
58 functional maltotriose transporter *SeAGT1* gene, they are unable to grow on α-oligoglucosides, but
59 expression of *S. cerevisae* regulator *ScMAL13* was sufficient to restore growth on trisaccharides. We
60 hypothesized that *S. pastorianus* maltotriose phenotype results from regulatory interaction between
61 *S.cerevisae* maltose transcription activator and the promoter of *SeAGT1*. We experimentally
62 confirmed the heterotic nature of the phenotype and thus this results provide experimental evidence
63 of the evolutionary origin of an essential phenotype of lager-brewing strains.

64

65 ## Introduction

66 *Saccharomyces pastorianus* is an interspecific hybrid of *S. cerevisiae* and *S. eubayanus* (1-4). *S.*
67 *pastorianus* strains are widely used for production of lager beer, which is currently the most
68 produced alcoholic beverage worldwide. Lager brewing requires alcoholic fermentation at relatively
69 low temperatures. *S. pastorianus* was hypothesized to have emerged by spontaneous hybridization
70 and to have colonized early lager-brewing processes due to a combination of cold-tolerance
71 inherited from *S. eubayanus* and superior fermentation kinetics inherited from *S. cerevisiae* (5-7).
72 Lager beer is brewed from barley wort, whose sugar composition consists, by weight, of
73 approximately 15% glucose, 60% maltose and 25% maltotriose (8). During wort fermentation,
74 maltotriose is generally only utilized after glucose and maltose are depleted, while its consumption is
75 also relatively slow and often incomplete (9-11).

76 Complete sugar utilization is desirable for lager beer fermentation to optimize concentrations of
77 ethanol and flavor compounds and to avoid residual sweetness (12). While *S. pastorianus* and *S.*
78 *cerevisiae* strains are capable of consuming maltotriose, none of the wild isolates of *S. eubayanus*
79 characterized thus far have been shown to possess this trait (6, 13, 14). These observations led to the
80 hypothesis that the ability of *S. pastorianus* to ferment maltotriose was inherited from *S. cerevisiae*
81 (6, 13, 15-17).

82 The genetic information for maltose utilization is well conserved in *Saccharomyces* species and
83 depends on three gene families. *MALT* genes encode plasma-membrane proton symporters with
84 varying substrate specificities and affinities (18, 19), *MALS* genes encode α-glucosidases that
85 hydrolyze α-oligoglucosides into glucose, while *MALR* genes encode a regulator required for
86 transcriptional induction of *MALT* and *MALS* genes by maltose (20, 21). In *Saccharomyces* species,
87 maltose-utilization genes are generally organized in *MAL* loci. These loci contain a *MALT* gene (called
88 *ScMALx1* and *SeMALTx* in *S. cerevisiae* and *S. eubayanus*, respectively), a *MALS* gene referred to as
89 *ScMALx2* or *SeMALSx* and a *MALR* gene referred to as *ScMALx3* or *SeMALRx* (13, 22). In the absence

90 of glucose and presence of maltose, the MalR regulator binds a bi-directional promoter, thereby
91 simultaneously activating expression of *MALT* and *MALS* genes (23).

92 The Sc*MAL1*-Sc*MAL4* and Sc*MAL6* loci of *S. cerevisiae* as well as the *SeMAL1-SeMAL4* loci of *S.*
93 *eubayanus* are located in subtelomeric regions (13, 24-26). While all *S. cerevisiae Sc*Malx1
94 transporters transport maltose, only *Sc*Mal11 is able to also transport maltotriose (9). *ScMAL11* (also
95 known as *ScAGT1*) shares only 57% nucleotide identity with other *ScMALx1* genes (27). The four
96 *SeMALT* (*SeMALT1-4*) genes identified in the genome of the Patagonian type strain FM1318/CBS
97 12357 of *S. eubayanus* were shown to encode functional maltose transporters, but none of these
98 genes enabled maltotriose transport (13). While no clear *ScAGT1* ortholog was found in *S. eubayanus*
99 CBS 12357^{T}, such an ortholog was recently found in the genomes of two North American isolates
100 assigned to the Holarctic subclade of *S. eubayanus* (14).

101 *S. pastorianus* inherited *MAL* genes from both *S. cerevisiae* and *S. eubayanus* (2, 4, 28). However, the
102 *S. cerevisiae*-derived maltotriose- transporter gene *ScAGT1* is truncated and, therefore, non-
103 functional in *S. pastorianus* (10). Instead, maltotriose consumption by *S. pastorianus* strains was
104 attributed to *SeAGT1* and *SpMTY1*/*SpMTT1* genes (29-32). In *S. pastorianus*, *SeAGT1* is located on *S.*
105 *eubayanus* CHRXV and was therefore, already before the identification of an *AGT1* ortholog in
106 Holarctic *S. eubayanus* strains (14), assumed to originate from *S. eubayanus* (2). *SpMTY1,* also
107 referred to as *SpMTT1,* is located on *S. cerevisiae* CHRVII and has less than 92% sequence identity
108 with other *Saccharomyces* maltose transporters (30). However, *SpMTY1* contains sequence patches
109 with high similarity to maltose transporters from *S. eubayanus* and *S. paradoxus* (33). Recently, two
110 independent laboratory evolution studies with *S. eubayanus* demonstrated that recombination of
111 different *SeMALT* genes yielded chimeric, neo-functionalized genes that encoded maltotriose
112 transporters (34, 35). *SpMTY1* may have resulted from successive introgressions of maltose-
113 transporter genes from *S. cerevisiae*, *S. eubayanus* and *S. paradoxus*.

114 Recently made *S. cerevisiae* x *S. eubayanus* laboratory hybrids showed similar lager-brewing
115 performance as *S. pastorianus* strains, also with respect to maltotriose utilization (6, 16, 17, 36). In
116 these hybrids, maltotriose consumption depended on the presence of a functional *Sc*Agt1
117 transporter encoded by the *S. cerevisiae* subgenome (37). However, in view of the non-functionality
118 of *ScAGT1* in current *S. pastorianus* strains, these laboratory hybrids did not fully recapitulate the
119 genetic landscape of *S. pastorianus* with respect to maltotriose fermentation (2, 6, 36).

120 Studies on laboratory hybrids based on *S. eubayanus* strains whose genomes are more closely related
121 to the *S. eubayanus* subgenome of *S. pastorianus* strains than that of the Patagonian type strain
122 CBS12357 might generate new insights into the evolution of maltotriose utilization in *S. pastorianus*.
123 To date, Himalayan *S. eubayanus* isolates show the highest sequence identity with the *S. eubayanus*
124 sub-genome of *S. pastorianus*, with up to 99.82% identity, as opposed to 99.56% for *S. eubayanus*
125 CBS12357^{T} (38).

126 Here, we investigated if and how the genomes of Himalayan *S. eubayanus* strains could have
127 contributed to maltotriose utilization in the earliest hybrid ancestors of current *S. pastorianus*
128 strains. To this end, we generated chromosome-level genome assemblies of these strains by long-
129 read DNA sequencing. Since the Himalayan strains were unable to utilize maltotriose, we functionally
130 characterized the assembled *MAL* genes and identified genetic determinants that prevented
131 maltotriose utilization. Subsequently, a laboratory hybrid of a representative Himalayan *S. eubayanus*
132 strain and a maltotriose-deficient ale strain of *S. cerevisiae* was generated to investigate the genetics
133 of maltotriose utilization in a hybrid context. We discuss the implications of the experimental results
134 for the proposed role and origin of *SeAGT1* in *S. pastorianus* and for the potential of hybridization to
135 enable maltotriose consumption in novel *Saccharomyces* hybrids.

136 ## Results

137 **Sequencing of Himalayan *S. eubayanus* strains revealed variations of sub-telomeric regions and**
138 **presence of novel putative maltose transporter genes.**

139 It has been proposed that the *S. eubayanus* genetic pool of *S. pastorianus* was inherited from an
140 ancestor of the Asian *S. eubayanus* lineage (38). With 99.82% identity, the Himalayan *S. eubayanus*
141 strains CDFM21L.1 and ABFM5L.1 that belong to the Holarctic lineage (39), present the closest
142 characterized relatives of the *S. eubayanus* ancestor of lager brewing yeasts. However, this distance
143 was based on a limited sequencing space (38) and the analysis did not investigate the presence of
144 specific *S. eubayanus* genetic markers found in *S. pastorianus* hybrids. Therefore, the genome of the
145 Himalayan *S. eubayanus* strain CDFM21L.1 was sequenced with a combination of long-read and
146 short-read techniques (Oxford Nanopore MinION and Illumina technologies, respectively) to
147 generate a near-complete draft reference genome sequence. The resulting CDFM21L.1 genome
148 assembly comprised 19 contigs, including its mitochondrial genome. All chromosomes were
149 completely assembled from telomere to telomere, except for chromosome XII, which was
150 fragmented into 3 contigs due to the repetitive rDNA region and manually assembled into a single
151 scaffold. With a total size of 12,034,875 bp, this assembly represents the first near-complete draft
152 genome of a *S. eubayanus* strain of the Holarctic clade (39).

153 Chromosome-level assemblies were hitherto only available for the Patagonia B-clade strain CBS
154 12357^{T} (1, 13). We identified three major structural differences in CDFM21L.1 relative to CBS12357^{T}
155 using Mauve (40): (i) a paracentric inversion in the sub-telomeric region of chromosome VII, involving
156 approximately 8 kbp, (ii) a translocation of approximately 12 kbp from the left sub-telomeric region
157 of chromosome VIII to the right sub-telomeric region of chromosome VI, and (iii) a reciprocal
158 translocation between approximately 20 kbp from the right sub-telomeric region of chromosome V
159 and approximately 60 kbp from the center of chromosome XII (Figure 1A). All structural variation
160 involved sub-telomeric regions, in accordance with their known relative instability (41-43).

161 An alignment comparison of the CDFM21L.1 and CBS 12357^T genomes with MUMmer revealed that
162 557 kb were unique to CDFM21L.1 and reciprocally 428 kb were unique to CBS 12357^T. Sequences
163 unique to CBS 12357^T (3.6% of its genome) and to CDFM21L.1 (4.6% of its genome) were located
164 primarily in sub-telomeric regions and in repetitive regions, such as rDNA on chromosome XII (Figure
165 1B). Out of the 32 sub-telomeric regions, 23 exhibited absence of synteny. Conserved synteny was
166 observed for sub-telomeric regions on CHRIII (left), CHRIV (left and right), CHRVI (left), CHRIX (right),
167 CHRXI (right), CHRXII (right), CHRXIV (left) and CHRXV (right) (Supplementary File 1).
168 The 428 kb of sequence that were absent in the Himalyan *S. eubayanus* strain included 99 annotated
169 ORFs (Supplementary File 1). Of the 99 ORFs that were (partly) affected, 11 were completely absent
170 in CDFM21L.1, involving mostly genes implicated in iron transport facilitation (Supplementary File 1).
171 The 557 kb of sequence that was not present in CBS 12357^T included 113 annotated ORFs
172 (Supplementary File 1). Of these 113 ORFs, 15 were completely absent in CBS 12357^T. These 15 ORFs
173 showed an overrepresentation of genes involved in transmembrane transport (Fishers exact test, P-
174 value 4.8E^{-5}) (Figure 1C).
175 Of the 15 ORFs unique to CDFM21L.1, three were identical orthologs of *S. cerevisiae MAL11/AGT1*.
176 These three ORFs were found in the sub-telomeric regions of chromosomes VII, XIV and XV. Their
177 sequence similarity with the *S. cerevisiae* CEN.PK113.7D and *S. pastorianus* CBS 1483 *MAL11/AGT1*
178 genes was 82.7% and 99.89%, respectively. In addition to these *SeAGT1* genes, CDFM21L.1 genome
179 sequence harbored three maltose transporters (*SeMALTx*), two maltases (*SeMALSx*) and three
180 regulators (*SeMALRx*) encoding genes. In contrast to the situation in *S. eubayanus* CBS 12357^T, none
181 of the *SeMAL* genes formed a canonical *MAL* locus in CDFM21L.1 (Figure 2). A systematic sequence
182 inspection of these CDFM21L.1 *SeMAL* genes revealed mutations that prematurely interrupted the
183 reading frames of *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and *SeMALT*3 (CHRXII).
184 In addition to *S. eubayanus* CDFM21L.1 strain, a second Himalayan *S. eubayanus* isolate (ABFM5L.1)
185 was sequenced. These two strains were 99.97% genetically identical at the nucleotide level, their
186 *MAL* genes were syntenic and the premature stop codons in *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and

187 *SeMALT*3 (CHRXII) were conserved. Two additional mutations were identified in one of the three
188 *SeAGT1* genes. A nucleotide variation on position 53 and 939 (T instead of an A and A instead of a G))
189 resulted in a glycine to valine and arginine to lysine change, respectively.

190 **Paradoxically, Himalayan *S. eubayanus* strains do not utilize maltose and maltotriose.**

191 Identification of *SeAGT1* in the two Himalayan *S. eubayanus* strains suggests an ability to not only
192 grow on maltose but also on maltotriose. Strains from the Holarctic clade have previously been
193 hypothesized to be the donor of the *S. eubayanus* sub-genome in *S. pastorianus* hybrids (38, 39).
194 However, no physiological data regarding their ability to grow on the sugars present in wort are
195 available. To assess their growth characteristics, the *Asian S. eubayanus* strains CDFM21L.1 and
196 ABFM5L.1, the Patagonian *S. eubayanus* type strain CBS 12357^{T} and the *S. pastorianus* strain CBS
197 1483 were grown on diluted industrial brewer's wort at 12 ˚C. As reported previously, *S. pastorianus*
198 strain CBS 1483 could utilize all three sugars but did not fully consume maltotriose (Figure 3) (11).
199 Also in accordance with previous observations (6), CBS 12357^{T} consumed glucose and maltose
200 completely, but left maltotriose untouched. However, in marked contrast to *S. eubayanus* CBS
201 12357^{T}, neither CDFM21L.1 nor ABFM5L.1 consume maltose after growth on glucose. Moreover, like
202 CBS 12357^{T}, maltotriose was not metabolized by these two *S. eubayanus* strains. While in CBS 12357^{T}
203 an ability to grow on maltose and an inability to grow on maltotriose could be readily attributed to its
204 *MAL* genes complement, CDFM21L.1 and ABFM5L.1 failed to grow on maltose even though they
205 appeared to contain complete genes encoding maltose (*SeMALT1* and *SeAGT1*) and maltotriose
206 (*SeAGT1*) transporters.
207

208 **Growth defects on maltose and maltotriose are caused by deficiency of the regulatory *Se*MalR**
209 **proteins in *S. eubayanus* CDFM21L.1.**

210 The recent characterization of maltose metabolism in CBS 12357^{T} showed that the coding regions of
211 transcriptionally silent maltose-transporter genes in *S. eubayanus* can potentially encode functional

212 proteins (13). The inability of the *S. eubayanus* Himalayan isolates to grow on α-oligosaccharides
213 precluded direct testing of transporter-gene functionality by deletion studies. Instead, these genes
214 were expressed in *S. cerevisiae* IMZ616, which is devoid of all native maltose metabolism genes (44).
215 The CDFM21L.1 transporter genes *SeMALT1, SeMALT2, SeMALT3* or *SeAGT1* were integrated at the
216 *ScSGA1* locus in IMZ616 along with the *S. cerevisiae* maltase gene *ScMAL12* (13), yielding a series of
217 strains overexpressing a single transporter (IMX1702 (*SeMALT1*), IMX1704 (*SeMALT2*), IMX1706
218 (*SeMALT3*) and IMX1708 (*SeAGT1*)). These strains, as well as the negative and positive control strains
219 IMZ616 and IMX1365 (IMZ616 expressing *ScAGT1* and *ScMAL12*), were grown on SM media
220 supplemented with either maltose or maltotriose. On maltose, not only the positive control strain
221 IMX1365, but also IMX1702 (*SeMALT1*) and IMX1708 (*SeAGT1*) were able to grow on maltose,
222 consuming 30 and 60%, respectively, of the initially present maltose after 100 h (Figure 4A). As
223 anticipated, the *SeMALT2* and *SeMALT3* alleles with premature stop codons did not support growth
224 on maltose. Of the two strains that grew on maltose, only IMX1708 (*SeAGT1*) also grew on
225 maltotriose. These results demonstrate that *SeAGT1* from a Holarctic *S. eubayanus* encoded a
226 functional maltotriose transporter and, consequently, that the inability of Holarctic strains to grow
227 on maltose and maltotriose was not caused by transporter dysfunctionality.
228 In addition to transport, metabolism of α-oligoglucosides requires maltase activity. Functionality of
229 the putative *SeMALS1* and *SeMALS2* maltase genes was tested by constitutive expression in strain
230 IMZ616, together with a functional *Sc*MAL31 transporter genes, yielding strains IMZ752 and IMZ753,
231 respectively. The maltase-negative strain IMX1313 was used as negative control. In SM medium with
232 maltose, both IMZ752 (*SeMALS1*) and IMZ753 (*SeMALS2*) grew and completely consumed maltose
233 within 65 h, demonstrating functionality of both hydrolase genes (Figure 4C).
234 In *S. cerevisiae* transcriptional regulation of *MALx2* and *MALx1* genes is tightly controlled by a
235 transcription factor encoded by *MALx3* genes. Malx3 binds an activating site located in the
236 bidirectional promoters that control expression of *MALx2* and *MALx1* genes (45, 46). To test whether
237 absence of maltose consumption in Himalayan *S. eubayanus* strains was caused by a lack

238 transcriptional upregulation of *SeMALT* and *SeMALS*, the *S. cerevisiae ScMAL13* gene was integrated
239 at the *SeSGA1* locus in *S. eubayanus* CDFM21L.1, under the control of a constitutive *ScPGK1*
240 promoter and *ScTEF2* terminator. *ScMAL13* expression in CDFM21L.1 enabled growth on maltose and
241 maltotriose (Figure 5A), indicating that a lack of transcriptional upregulation was indeed the cause of
242 the parental strain's inability to grow on these oligoglucosides. However, consumption of maltose
243 and maltotriose was incomplete and consumed sugars were almost exclusively respired, as no
244 ethanol was measured after 60 h of cultivation.

245 The possibility to grow an engineered variant of *S. eubayanus* CDFM21L.1 on α-oligoglucosides
246 offered an opportunity to study transporter function in its native context. Complementary functional
247 characterization by gene deletion of *SeMALT1* and *SeAGT1* was performed using CRISPR-Cas9
248 genome editing method (13, 47). Deletion of *SeMALT1* and *SeAGT1* in CDFM21L.1 resulted in strains
249 IMK820 and IMK823, respectively. Complete deletion of *SeAGT1* required disruption of six alleles. To
250 confirm the complete removal of all copies, the genome of IMK823 was sequenced. Mapping reads
251 onto the reference *S. eubayanus* CDFM21L.1 genome assembly confirmed that all six alleles were
252 removed simultaneously. Subsequently, the regulator expression cassette (*ScPGK1*$_p$-*ScMAL13*-
253 *ScTEF2*$_t$) was integrated in IMK820 and IMK823 at the *SeSGA1* locus yielding strains IMX1939 and
254 IMX1940, respectively (Figure 5B). The four deletion strains IMK820 (*SemalT1*Δ), IMK823 (*Seagt1*Δ),
255 IMX1939 (*SemalT1*Δ *Sesga1*Δ::*ScMAL13*) and IMX1940 (*Seagt1*Δ *Sesga1*Δ::*ScMAL13*) were
256 characterized on SMG, SMM or SMMt. All four strains were able to grow on glucose (Supplementary
257 Figure 3). While strains IMK820, IMK823 and IMX1940 were unable to grow on maltose or
258 maltotriose (Figure 5C), strain IMX1939 (*SemalT1*Δ *Sesga1*Δ::*SeMAL13*), which harbored functional
259 *SeAGT1* copies, grew on maltose as well as on maltotriose. However, after 64 h of growth, these
260 sugars were only partially consumed. Only 1.2 g L^{-1} ethanol was produced from maltose and no
261 ethanol formation was observed during growth on maltotriose. The low ethanol concentration and
262 the relatively high OD_{660nm} suggest that, under the experimental conditions strain IMX1939 exhibited
263 a Crabtree negative phenotype and exclusively respired maltotriose. *S. eubayanus* IMX1940 (*Seagt1*Δ

264 *Sesga1*Δ::*SeMAL13*) did not consume maltotriose after 84 h of incubation. Moreover, despite the
265 presence of *SeMALT1*, which encoded a functional maltose transporter upon expression in *S.*
266 *cerevisiae* IMZ616, strain IMX1940 was also unable to consume maltose.

267 In addition to a functional Malx3 transcription factor, transcriptional activation of *MAL* genes also
268 requires presence of a cis-regulatory motif in the promoter of regulated genes. Transcriptome
269 analysis of *S. eubayanus* CBS 12357^{T} recently showed that absence of a canonical cis-regulatory motif
270 in *SeMALT1* and *SeMALT3* of *S. eubayanus* CBS 12357^{T} caused a deficiency in their expression (13). To
271 further explore regulation of *SeMAL* and *SeAGT1* genes, we investigated the impact of carbon
272 sources on genome-wide transcriptome and, specifically, on transcriptional activation of genes
273 involved in maltose metabolism. Duplicate cultures of *S. eubayanus* strain IMX1765 (*ScPGK1*$_{p}$-
274 *ScMAL13-ScTEF2*$_{t}$) were grown on SMG, SMM and SMMt at 20 °C and sampled in mid-exponential
275 phase. After mRNA isolation and processing, cDNA libraries and reads were assembled onto the
276 newly annotated *S. eubayanus* CDFM21L.1 genome to calculate FPKM (fragments per kilobase of
277 feature (gene) per million reads mapped) expression values. The heterologous regulator *ScMAL13*,
278 expressed from the constitutive *ScPGK1* promoter, displayed the same expression in glucose- and
279 maltose-grown cultures. Although *ScMAL13* was efficiently expressed on glucose, none of the nine *S.*
280 *eubayanus* maltose genes (the three identical *SeAGT1* copies being undistinguishable) were
281 transcriptionally induced under these conditions (Figure 6), confirming that the hierarchical
282 regulatory role of glucose catabolite repression (45, 48) also takes place in *S. eubayanus*. During
283 growth on maltose, all nine genes were significantly upregulated relative to glucose-grown cultures
284 but large variations in expression level were observed. The maltase genes *SeMALS1* and *SeMALS2*
285 and the transporter gene *SeAGT1* showed the highest upregulation, with fold changes of 148, 161
286 and 2355 respectively. Although upregulated *SeMALT1* displayed a fold change of 13, its normalized
287 expression in maltose-grown cultures was 886-fold lower than that of *SeAGT1*. This weaker
288 upregulation might explain why, despite the ability of its coding region to support synthesis of a
289 functional maltose transporter, *SeMALT1* alone could not restore growth on maltose. The

290 transcriptome data also revealed that the absence of maltose induction in CDFM21L.1 was not
291 associated with defective cis-regulatory elements in *SeMALR* promoter sequences since the regulator
292 genes were properly activated, instead these results would suggest that the *Se*MalR regulator are not
293 functional.

294 **Hybridization of two maltotriose-deficient *S. eubayanus* and *S. cerevisiae* lineages results in**
295 **heterosis through regulatory crosstalk.**

296 The genetic make-up of *S. pastorianus* lager-brewing yeasts strongly advocates that they originate
297 from hybridization of *S. cerevisiae* and *S. eubayanus* parental lineages that were both unable to
298 metabolize maltotriose (2). This hypothesis is consistent with the recurrent mutation in the *S.*
299 *cerevisiae AGT1* allele of *S. pastorianus* strains as well as with the inability of Himalayan strains of *S.*
300 *eubayanus* to grow on these oligoglucosides.

301 Spores of the Himalayan *S. eubayanus* CDFM21L.1 were hybridized with *S. cerevisiae* CBC-1. This top-
302 fermenting *S. cerevisiae* is recommended for cask and bottle conditioning and unable to consume
303 maltotriose (Lallemand, Montreal, Canada). Analysis of the CBC-1 assembly, obtained by a
304 combination of long and short read sequencing, linked its maltotriose-negative phenotype to a total
305 absence of the *MAL11/AGT1* gene. The resulting laboratory interspecific hybrid HTSH020 was
306 characterized at 12 °C on synthetic wort, a defined medium whose composition resembles that of
307 brewer's wort. While *S. eubayanus* CDFM21L.1 only consumed glucose and *S. cerevisiae* CBC-1
308 consumed glucose and maltose after 103 h (Figure 7A), the interspecific hybrid HTSH020 completely
309 consumed glucose, maltose and partially consumed maltotriose after 105 h, thus resembling
310 characteristics of *S. pastorianus* strains (e.g. CBS1483 (11)). In addition to this gain of function, the
311 hybrid HTSH020 outperformed both its parents on maltose consumption, since it depleted this sugar
312 in 70 h instead of 95 h for strain CBC-1. Since *S. cerevisiae* grows generally slower at 12 °C, the
313 experiments were also performed at 20 °C where HTSH020 consumed all maltose 16 h earlier than
314 CBC-1 (Supplementary Figure 4).

315 Transcriptome analysis of the hybrid strain HTSH020 grown on SM with different carbon sources
316 showed that *SeAGT1* expression was repressed during growth on glucose, with a normalized
317 expression level of 7 FPKM (Figure 7B). When grown on SM maltose, *SeAGT1, SeMALS1* and *SeMALS2*
318 were significantly induced, with fold increases of 816, 109 and 116, respectively (Figure 7B). Although
319 *SeMALT1* and *SeMALT2* were induced, these transporters do not contribute to maltose metabolism
320 due to truncation of their ORFs. These transcriptome data demonstrated that *SeAGT1* and *SeMALS*
321 genes are induced by regulatory crosstalk between regulators encoded from the CBC-1 *S. cerevisiae*
322 sub-genome and maltotriose transporter genes harbored by the *S. eubayanus* genome. This
323 laboratory hybridization experiment may be the closest reproduction yet of how, centuries ago,
324 maltotriose-fermentation capacity arose in the first hybrid ancestor of *S. pastorianus*.
325

326 ## Discussion

327 The ability to consume maltose and maltotriose represents a key performance indicator of *S.*
328 *pastorianus* lager-brewing strains (10). This study demonstrates how mating of *S. cerevisiae* and *S.*
329 *eubayanus* strains that cannot themselves ferment maltotriose, can yield maltotriose-fermenting
330 hybrids. This laboratory study illustrates how, centuries ago, maltotriose-fermentation capacity may
331 have arisen in the first hybrid ancestor of *S. pastorianus*.

332 While the origin of the *S. eubayanus* parent of *S. pastorianus* strains is still under debate (49-
333 51), phylogenetic analysis suggested a Far East Asian origin (38). However, this interpretation was
334 based on a limited sequencing space and was constrained by the quality of available sequence
335 assemblies. Since an ortholog of *SeAGT1* had previously only been found in the *S. eubayanus* sub-
336 genome of *S. pastorianus* strains, this finding revived the discussion on the geographical origin of the
337 ancestral *S. eubayanus* parent (14). The high-quality, annotated genome assemblies of the Himalayan
338 *S. eubayanus* strains CDFM21L.1 and ABFM5L.1 presented in the present study revealed several
339 copies of *SeAGT1*, whose very high sequence identity with *S. pastorianus SeAGT1* are consistent with

340 the previously proposed Asian origin of the S. *eubayanus* sub-genome of *S. pastorianus* (14, 38, 39).
341 Next, genome-sequence comparison of the Patagonian B sub-clade *S. eubayanus* strain CBS12357^T
342 and the Holarctic sub-clade strain CDFM21L.1 revealed homoplasy of *SeAGT1*, probably reflecting
343 that these sub-clades evolved in different ecological niches.

344 The *S. eubayanus* wild stock whose genome sequence most closely corresponds to the *S.*
345 *eubayanus* sub-genome of *S. pastorianus* originates from the Tibetan plateau of the Himalaya (38).
346 However, the first *S. cerevisiae x S. eubayanus* hybrid, from which current lager yeasts evolved by
347 centuries of domestication, likely originates from a region between Bavaria and Bohemia in Central
348 Europe. So far, European *S. eubayanus* isolates have not been reported. This may indicate that the
349 original hybridization event occurred elsewhere or that the ancestral European lineage went extinct.
350 The recent detection, in a metagenomics analysis of samples from the Italian Alps, of ITS1 sequences
351 corresponding to *S. eubayanus* could indicate that a wild European lineage exists after all (52).

352 Functional characterization by heterologous complementation of an *S. cerevisiae* mutant
353 strain established that the *Se*Agt1 transporters from the Himalayan *S. eubayanus* strains CDFM21L.1
354 and ABFM5L supported uptake of maltose and maltotriose. After showing that these strains also
355 encoded a functional maltase gene, their inability to grow on maltose and maltotriose was attributed
356 to an inability to transcriptionally upregulate maltose metabolism genes likely caused by loss of
357 function mutations the regulator. In *S. cerevisiae* and, to some extent, ,in *S. eubayanus* strains of the
358 Patagonian B sub-clade such as CBS12357^T (13, 26, 53), MAL loci exhibit a specific organization in
359 which a transporter (*MALT*) and a hydrolase (*MALS*) gene are expressed from the same bidirectional
360 promoter and are located adjacent to a regulator gene (*MALR*) (21). In contrast, of the seven
361 genomic regions harboring *MAL* genes in the two Asian *S. eubayanus* strains, none showed this
362 canonical organization (Figure 2) and the subtelomeric regions carrying *SeAGT1* did not harbour
363 sequences similar to hydrolase or regulator genes. Subtelomeric regions harboring the other *MAL*
364 genes indicated intensive reorganization as a result of recombination. In particular, subtelomeric
365 regions on CHRII, CHRV and CHRXII provide clear indications for recombination events that scattered

366 genes from ancestral MAL1 and MAL2 loci over several chromosomes. A similar interpretation could
367 explain the reorganization MAL3 on CHRXIII (Figure 2). Similar events may have contributed to loss of
368 function of the MAL regulators (MalR), as exemplified by the occurrence of a non-synonymous
369 mutation in S*eMALR1* resulting in loss of function. These rearrangements did, however, not
370 inactivate the *cis*-regulatory sequences of the *MAL* genes, since complementation with a functional
371 *ScMAL13* allele caused induction of most *SeMAL* genes (Figure 6, Figure 7B) and, thereby, the
372 heterotic maltotriose-positive phenotype of the hybrid strain HTSH020. Together with the high copy
373 number of *SeAGT1*, this heterotic complementation may have been the main driver for colonization
374 of low-temperature brewing processes by the early hybrid ancestors of current *S. pastorianus* strains.
375 Recent work on adaptation to brewing environments of laboratory *S. cerevisiae* × *S. eubayanus*
376 hybrids showed loss maltotriose utilization during serial transfer in wort (37). A similar loss of
377 maltotriose utilization is frequently encountered in *S. cerevisiae* ale strains (54), as well as in some
378 Saaz-type *S. pastorianus* strains (55). This is thus in contrast with retention of a maltotriose
379 assimilation phenotype by Frohberg-type *S. pastorianus* strains. This may have been facilitated by the
380 occurrence of multiple copies of the *SeAGT1* gene in the *S. eubayanus* ancestor, which could act as a
381 sequence buffer to counteracting adverse effects of gene copy loss. The recent release of the first
382 long-read sequencing assembly of *S. pastorianus* enabled a precise chromosomal mapping of the
383 maltose-metabolism genes (56) and showed that the Frohberg type *S. pastorianus* strains CBS 1483
384 harbored one copy of *SeAGT1* on the *S. eubayanus* CHRXV section (as in CDFM21L.1) of the chimeric
385 chromosome formed from *Se*CHRXV and *Se*CHRVIII (56).
386 Differential retention and loss of maltotriose consumption in *S. pastorianus* lineages may reflect
387 different brewing process conditions during domestication. In modern brewing processes based on
388 high-gravity wort, cell division is largely constrained to the glucose and maltose phases, which occur
389 before depletion of nitrogen sources (57). It may be envisaged that, in early lager-brewing processes,
390 unstandardized mashing processes generated wort with a higher maltotriose content, which would
391 have allowed for continued yeast growth during the maltotriose consumption phase. During serial

392 transfer on sugar mixtures, the selective advantage of consuming a specific sugar from a mixture
393 correlates with the number of generations on that sugar during each cycle (58, 59). Such conditions
394 would therefore have conferred a significant selective advantage to a maltotriose-assimilating *S.*
395 *cerevisiae* x *S. eubayanus* hybrid, especially if, similar to current ale yeasts, the *S. cerevisiae* parent
396 was unable to ferment maltotriose.

397 The heterotic phenotype that was reconstructed in the interspecies *S. cerevisiae* x *S.*
398 *eubayanus* hybrid HTS020 resulted from combination of dominant and recessive genetic variations
399 from both parental genomes. *S. eubayanus* contributed the *SeAGT1* gene and its functional *cis*-
400 regulatory sequences, but also harbored recessive mutations in *MALR* genes that allowed full
401 expression of the heterotic phenotype. These mutations were complemented with a set of *S.*
402 *cerevisiae* genes including a functional *MALR* and a non-functional *ScAGT1* gene to match the
403 mutations found in *S. pastorianus* (2). Although some *S. pastorianus* strains harbor an additional
404 maltotriose transporter encoded by *SpMTT1 (30)*, this gene was recently proposed to have emerged
405 after the original hybridization event as a result of repeated recombination between *MALT* genes
406 from both sub-genomes (33).

407 Maltotriose fermentation is likely not the only heterotic phenotype of S. *pastorianus* strains.
408 Flocculation or formation of complex aroma profiles (28, 60) are phenotypes that are not fully
409 understood and difficult to reproduce, that also might result from heterosis (37).

410 Laboratory-made *S. cerevisiae* x *S. eubayanus* hybrids hold great potential for brewing process
411 intensification and for increasing product diversity. In addition to increasing our understanding of the
412 evolutionary history of lager yeast genomes evolutionary, this study has implications for the design
413 of new hybrids. Hitherto, laboratory crosses of *S. cerevisiae* x *S. eubayanus* strains were designed
414 based on combination of dominant traits of the parental strains. Our results show that recessive
415 traits can be just as important as contributors to the genetic diversity of such hybrids.

416 ## Materials and methods

417 **Strains and maintenance**

418 All strains used in this study are listed in Table 1. Stock cultures of *S. eubayanus* and *S. cerevisiae*
419 strains were grown in YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose) until late
420 exponential phase, complemented with sterile glycerol to a final concentration of 30 % (v/v) and
421 stored at -80 °C as 1 mL aliquots until further use.

422 **Media and cultivation**

423 *S. eubayanus* batch cultures were grown on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g
424 L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4.7H_2O$, 1 mL L^{-1} trace element solution, and 1 mL L^{-1} vitamin solution
425 (61). The pH was set to 6.0 with 2 M KOH prior to autoclaving at 120 °C for 20 min. Vitamin solutions
426 were sterilized by filtration and added to the sterile medium. Concentrated sugar solutions were
427 autoclaved at 110 °C for 20 min or filter sterilized and added to the sterile flasks to give a final
428 concentration of 20 g L^{-1} glucose (SMG), maltose (SMM) or maltotriose (SMMt). With the exception
429 of IMZ752 and IMZ753, *S. cerevisiae* batch cultures were grown on SM supplemented with 150 mg L^{-1}
430 uracil (62) to compensate for loss of plasmid pUDC156 that carried the *Spcas9* endonuclease gene,
431 and supplemented with 20 g L^{-1} glucose (SM_uG), maltose (SM_uM) or maltotriose (SM_uMt). All batch
432 cultures were grown in 250 mL shake flasks with a working volume of 50 mL. The cultures were
433 inoculated at an initial OD_{660nm} of 0.1 and incubated under an air atmosphere and shaken at 200 rpm
434 and at 20 °C in a New Brunswick™ Innova44 incubator (Eppendorf Nederland B.V, Nijmegen, The
435 Netherlands).

436 *S. eubayanus* strains transformed with plasmids pUDP052 ($gRNA_{SeSGA1}$), pUDP091 ($gRNA_{SeMALT1}$) and
437 pUDP090 ($gRNA_{SeAGT1}$) were selected on modified SMG medium ($SM_{Ace}G$) in which $(NH_4)_2SO_4$ was
438 replaced by 6.6 g L^{-1} K_2SO_4 and 10 mM acetamide (63). SM-based solid media contained 2 % Bacto
439 Agar (BD Biosciences, Franklin Lakes, NJ). *S. cerevisiae* strains expressing either *SeMALT, SeMALS* or
440 *ScMALR* were selected on $SM_{Ace}G$. For plasmid propagation, *E. coli* XL1-Blue-derived strains (Agilent
441 Technologies, Santa Clara, CA) were grown in Lysogeny Broth medium (LB, 10 g L^{-1} tryptone, 5 g L^{-1}

442 yeast extract, 5 g L^{-1} NaCl) supplied with 100 mg L^{-1} ampicillin. Synthetic wort medium (SWM) for
443 growth studies contained 14.4 g·L^{-1} glucose, 2.3 g·L^{-1} fructose, 85.9 g·L^{-1} maltose, 26.8 g·L^{-1}
444 maltotriose, 5 g·L^{-1} $(NH_4)_2SO_4$, 3 g·L^{-1} KH_2PO_4, 0.5 g·L^{-1} $MgSO_4.7H_2O$, 1 mL·L^{-1} trace element solution, 1
445 mL·L^{-1} vitamin solution, supplemented with the anaerobic growth factors ergosterol and Tween 80
446 (0.01 g·L^{-1} and 0.42 g·L^{-1} respectively), as previously described (61).
447 Industrial wort (containing 14.4 g L^{-1} glucose, 85.9 g L^{-1} maltose, 26.8 g L^{-1} maltotriose, 2.3 g L^{-1}
448 fructose and 269 mg L^{-1} FAN) was provided by Heineken Supply Chain B.V. (Zoeterwoude, the
449 Netherlands). The wort was supplemented with 1.5 mg L^{-1} of Zn^{2+} by addition of $ZnSO_4.7H_2O$,
450 autoclaved for 30 min at 121 °C, filtered using Nalgene 0.2 µm SFCA bottle top filters (Thermo
451 Scientific) and diluted with sterile demineralized water. Sporulation medium consisted of 2 % (w/v)
452 KAc in MilliQ water set to pH 7.0 with KOH, autoclaved at 121 °C for 20 min.

453 **Microaerobic growth experiments**

454 Microaerobic cultures were grown in 250-mL airlock-capped Neubor infusion bottles (38 mm neck,
455 Dijkstra, Lelystad, Netherlands) containing 200 mL three-fold diluted industrial wort supplemented
456 with 0.4 mL L^{-1} Pluronic antifoam (Sigma-Aldrich, St. Louis, MO). Bottle caps were equipped with a 0.5
457 mm x 16 mm Microlance needle (BD Biosciences) sealed with cotton to prevent pressure build-up.
458 Sampling was performed aseptically with 3.5 mL syringes using a 0.8 mm x 50 mm Microlance needle
459 (BD Biosciences). Microaerobic cultures were inoculated at an OD_{660nm} of 0.1 from stationary-phase
460 precultures in 50 mL Bio-One Cellstar Cellreactor tubes (Sigma-Aldrich) containing 30 mL of the same
461 medium, grown for 4 days at 12 °C. Bottles were incubated at 12 °C and shaken at 200 rpm in a New
462 Brunswick Innova43/43R shaker (Eppendorf Nederland B.V.). At regular intervals, 3.5 mL samples
463 were collected in 24 deep-well plates (EnzyScreen BV, Heemstede, Netherlands) using a LiHa liquid
464 handler (Tecan, Männedorf, Switzerland) to measure OD_{660nm} and external metabolites. 30 µL of each
465 sample was diluted 5-fold in demineralized water in a 96 well plate and OD_{660nm} was measured with a
466 Magellan Infinite 200 PRO spectrophotometer (Tecan). From the remaining sample, 150 µL was

467 vacuum filter sterilized using 0.2 µm Multiscreen filter plates (Merck, Darmstadt, Germany) for HPLC
468 measurements.

469 **Analytical methods**

470 Optical densities of yeast cultures were measured with a Libra S11 spectrophotometer (Biochrom,
471 Cambridge, United Kingdom) at a wavelength of 660 nm. Biomass dry weight was measured by
472 filtering 10-mL culture samples over pre-weighed nitrocellulose filters with a pore size of 0.45 µm.
473 Filters were washed with 10 mL water, dried in a microwave oven (20 min at 350 W) and reweighed.
474 Sugars were measured using a high pressure liquid chromatography Agilent Infinity 1260 series
475 (Agilent Technologies) using a Bio-Rad Aminex HPX-87H column at 65 °C with 5 mM sulfuric acid at a
476 flow rate of 0.8 mL min^{-1}. Compounds were measured using a RID at 35 °C. Samples were centrifuged
477 at 13,000 g for 5 min to collect supernatant or 0.2 µm filter-sterilized before analysis.

478 **Plasmid construction**

479 Plasmids used and constructed in this study are listed in Table 2, oligonucleotide primers used in this
480 study are listed in Supplementary Table 1. Coding regions of *SeMALT1*, *SeMALT2*, *SeMALT3* and
481 *SeAGT1* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity DNA polymerase
482 (Thermo Scientific), according to the supplier's instructions with primers pairs 12355/12356,
483 12357/12358, 12359/12360 and 12361/12362, respectively. The coding sequence of *ScMAL31* was
484 amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo
485 Scientific), according to the supplier's instructions with primer pairs 9942/9943. Each primer carried a
486 40 bp extension complementary to the plasmid backbone of p426-TEF-amdS (64), which was PCR
487 amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and primers 7812/5921.
488 Each transporter fragment was assembled with the p426-TEF-amdS backbone fragment using
489 NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in plasmids pUD444
490 (*ScMAL31*), pUD794 (*SeMALT1*), pUD795 (*SeMALT2*), pUD796 (*SeMALT3*) and pUD797 (*SeAGT1*). All

491 plasmids were verified for correct assembly by Sanger sequencing (Baseclear, Leiden, The
492 Netherlands).

493 *SeMALS1* and *SeMALS2* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity
494 DNA polymerase (Thermo Scientific), with primers pairs 14451/14453 and 14452/14453,
495 respectively. Each primer pair carried a 30 bp extension complimentary to the plasmid backbone of
496 pUDE044 (65) which was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo
497 Scientific) and primers 14449/14450. Resulting amplicons were assembled using NEBuilder HiFi DNA
498 Assembly (New England Biolabs), resulting in plasmids pUDE843 (*SeMALS1*) and pUDE844 (*SeMALS2*)
499 that were verified by Sanger sequencing (Baseclear).

500 *S. cerevisiae ScMAL13*, the *ScPGK1* promoter and the *ScTEF2* terminator were amplified from
501 CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), with
502 primer pairs 12915/12916, 9421/9422 and 10884/10885, respectively. Fragments were gel purified
503 and used with pYTK074, pYTK082 and pYTK083 in Golden Gate assembly according to the yeast
504 toolkit protocol (66) resulting in pUDE780, which was verified by Sanger sequencing (Baseclear).

505 Guide-RNA (gRNA) sequences for deletion of *SeMALT1* and *SeAGT1* in CDFM21L.1 were designed as
506 described previously (47). The DNA sequences encoding these gRNAs were synthesized at GeneArt
507 (Thermo Scientific) and were delivered in pUD631 and pUD634, respectively. The gRNA spacer
508 sequences (Se*MALT1* 5' CCCCGATATTCTTTACACTA 3', Se*AGT1* 5'- AGCTTTGCGAAAATATCCAA -3') and
509 the structural gRNA sequence were flanked at their 5' ends by the Hammerhead ribozyme (HH) and
510 at their 3' ends by the Hepatitis Delta Virus ribozyme (HDV) (67). The HH-gRNA-HDV fragment was
511 flanked on both ends with a BsaI site for further cloning (47, 68). Plasmids pUDP091 ($gRNA_{SeMALT1}$) and
512 pUDP090 ($gRNA_{SeAGT1}$) were constructed by Golden Gate cloning by digesting pUDP004 and the gRNA-
513 carrying plasmid (pUD631 and pUD634, respectively) using BsaI and ligating with T4 ligase (69).
514 Correct assembly was verified by restriction analysis with PdmI (Thermo Scientific) and Sanger
515 sequencing (Baseclear).

516 **Strain construction**

517 *S. cerevisiae* IMZ616, which cannot grow on α-glucosides (44), was used as a host to test functionality
518 of individual *S. eubayanus* (putative) maltose transporter genes (13). *S. cerevisiae* IMX1702 was
519 constructed by integrating *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ and *ScTEF1*$_p$-*SeMALT1-ScCYC1*$_t$ at the *ScSGA1*
520 locus of strain IMZ616. A fragment containing the *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ transcriptional unit
521 was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) from pUDE044
522 with primers 9596/9355, which included a 5' extension homologous to the upstream region of the
523 *ScSGA1* locus and an extension homologous to the co-transformed transporter fragment,
524 respectively. The DNA fragment carrying the *S. eubayanus SeMALT1* maltose symporter (*ScTEF1*$_p$-
525 *SeMALT1-ScCYC1*$_t$) was PCR amplified from pUD794 using primers 9036/9039, which included a 5'
526 extension homologous to the co-transformed transporter fragment and an extension homologous to
527 the downstream region of the *ScSGA1* locus, respectively. To facilitate integration in strain IMZ616,
528 the two PCR fragments were co-transformed with plasmid pUDR119 (*amdS*), which expressed a gRNA
529 targeting *ScSGA1* (spacer sequence: 5'-ATTGACCACTGGAATTCTTC-3') (70). The plasmid and repair
530 fragments were transformed using the LiAc yeast transformation protocol (71) and transformed cells
531 were plated on $SM_{Ace}G$. Correct integration was verified by diagnostic PCR with primers pairs
532 4226/4224. Strains *S. cerevisiae* IMX1704, IMX1706 and IMX1708 were constructed following the
533 same principle, but instead of using pUD794 to generate the transporter fragment, pUD795, pUD796
534 and pUD797 were used to PCR amplify *ScTEF1*$_p$-*SeMALT2-ScCYC1*$_t$, *ScTEF1*$_p$-*SeMALT3-ScCYC1*$_t$ and
535 *ScTEF1*$_p$-*SeAGT1-ScCYC1*$_t$ respectively. IMX1313 was constructed in a similar way using only *ScTEF1*$_p$-
536 *ScMAL31-ScCYC1*$_t$ amplified with primer pair 9036/11018 which contain 5'and 3' extensions
537 homologous to the upstream and downstream region of the *ScSGA1* locus. Correct integration was
538 verified by diagnostic PCR with primer pair 4226/4224 (Supplementary Figure 1). All PCR-amplified
539 gene sequences were Sanger sequenced (BaseClear). IMX1313 was grown on YPD to loose pUDR119
540 (*URA3*) and pUDC156 (*amdS*). An isolate unable to grown on SMG without uracil and with acetamide

541 was selected and named IMX1313Δ. This strain was able to grow on SMG supplemented with 150 mg
542 L^{-1} uracil.

543 To assess functionality of CDFM21L.1 *SeMALS1*, IMX1313Δ was transformed with 100 ng pUDE843
544 (*ScTDH3*$_p$-*SeMALS1-ScADH1*$_t$) by electroporation (47), resulting in strain IMZ752. Transformants were
545 selected on SMG plates after 5 days of incubation at 20 °C and validated by PCR (DreamTaq
546 polymerase, Thermo Scientific) using primer pair 14454/14455 (Supplementary Figure 1). Similarly,
547 functionality of the *SeMALS2* maltase gene of CDFM21L.1 was assessed by transforming IMX1313Δ
548 with pUDE844 (*ScTDH3*$_{pr}$-*SeMALS2-ScADH1*$_{ter}$), resulting in strain IMZ753.

549 *S. eubayanus* IMK820 (*SemalT1Δ*) was constructed by transforming CDFM21L.1 with 200 ng of
550 pUDP091 and 1 µg of 120 bp repair fragment obtained by mixing an equimolar amount of primers
551 12442/12443, as previously described (47). As a control, the same transformation was performed
552 without including the repair DNA fragment. Transformants were selected on $SM_{Ace}G$ plates. *S.*
553 *eubayanus* IMK823 (*Seagt1Δ*) was constructed similarly, using pUDP090 and primer pair
554 11320/11321. Deletion of *SemalT1* was verified by PCR with primer pair 11671/11672 and Sanger
555 sequencing. The *Seagt1* deletion was verified by PCR using primer pair 12273/12274, and by Illumina
556 whole-genome sequencing and read alignment to the reference genome of CDFM21L.1 (Bioproject
557 accession number PRJNA528469).

558 Strains IMX1765, IMX1939 and IMX1940 were constructed by inserting *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ at
559 the *SeSGA1* locus of CDFM21L.1, IMK820 and IMK823, respectively. A repair fragment containing
560 *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ was amplified from pUDE780 with primer pair 12917/12918. Strains
561 CDFML21L.1, IMK820 and IMK823 were transformed by electroporation by addition of 350 ng of
562 repair fragment and 560 ng pUDP052 (*amdS*) into the cells as previously described (47).
563 Transformants were plated on $SM_{Ace}G$ and incubated at 20 °C. IMX1762 was constructed similarly
564 using a repair fragment with *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ amplified from pUDE044 with primer pair
565 12319/12320. Strains were verified by PCR using primer pair 12635/12636 and Sanger sequencing.

566 **Hybrid Construction**

567 The *S. cerevisiae* X *S. eubayanus* hybrid HTSH020 was constructed by spore-to-spore mating . The *S.*
568 *eubayanus* strain CDFM21L.1 and the *S. cerevisiae* strain CBC-1 were grown in 20 mL YPD at 20 °C
569 until late exponential phase. Cells were centrifuged for 5 min at 1000 g and washed twice in
570 demineralized water. Cells were re-suspended in 20 mL sporulation medium and incubated for 64 h
571 at 20 °C. Presence of spores was verified by microscopy. Asci were harvested by centrifugation for 5
572 min at 1000 g, and washed with demineralized water, resuspended in 100 µL demineralized water
573 containing 100 U/mL of Zymolyase (MP Bio, Santa Ana, CA) and incubated for 10 min at 30 °C. Spores
574 were washed and plated on the edge of a YPD agar plate. Spores from the two strains were brought
575 in contact with each other with an MSM System 400 micromanipulator (Singer Instruments, Watchet,
576 United Kingdom). Zygote formation was observed after 6-8 h. Emerging colonies were re-streaked
577 twice on SM 2% maltose at 12 °C. Successful hybridization was verified by multiplex PCR using
578 DreamTaq DNA polymerase (Thermo Scientific), by amplifying the *S. cerevisiae* specific *MEX67* gene
579 with primer pairs 8570/8571 and by amplifying the *S. eubayanus* specific gene *SeFSY1* with primers
580 8572/8573 (Supplementary Figure 2), as previously described (72).

581 **Illumina sequencing**

582 Genomic DNA of *S. eubayanus* strains CDFM21L.1 and ABFM5L.1, *S. cerevisiae* strain CBC-1 and *S.*
583 *cerevisiae* x *S. eubayanus* strain HTSH020 was isolated as previously described (4). Paired-end
584 sequencing (2 X 150 bp) was performed on a 350 bp PCR-free insert library using Illumina HiSeq2500
585 (San Diego, CA) by Novogene(HK) Company Ltd (Hong Kong, China). Genomic DNA of the strains CBC-
586 1 and HTSH020 was sequenced in house on a MiSeq sequencer (Illumina) with 300 bp paired-end
587 reads using PCR-free library preparation. Sequence data are available at NCBI under Bioproject
588 accession number PRJNA528469.

589 **MinION long read sequencing**

590 For long-read sequencing, a 1D sequencing library (SQK-LSK108) was prepared for CDFM21L.1 and
591 CBC-1 and loaded onto an FLO-MIN106 (R9.4) flow cell, connected to a MinION Mk1B unit (Oxford
592 Nanopore Technology, Oxford, United Kingdom), according to the manufacturer's instructions.
593 MinKNOW software (version 1.5.12; Oxford Nanopore Technology) was used for quality control of
594 active pores and for sequencing. Raw files generated by MinKNOW were base-called using Albacore
595 (version 1.1.0; Oxford Nanopore Technology). Reads with a minimum length of 1000 bp were
596 extracted in fastq format. For CDFM21L.1, 3.26 Gb of sequence with an average read length of 8.07
597 kb was obtained and for CBC-1 3.04 Gb sequence with an average read length of 7.27 kb. Sequencing
598 data are available at NCBI under Bioproject accession number PRJNA528469.

599 **De novo *assembly***

600 *De novo* assembly of the Oxford Nanopore MinION dataset was performed using Canu (v1.4, setting:
601 genomesize=12m) (73). Assembly correctness was assessed using Pilon (74) and further corrected by
602 "polishing" of sequencing/assembly errors by aligning Illumina reads with BWA (75) using correction
603 of only SNPs and short indels (–fix bases parameter). For HTSH020, an artificial reference genome
604 was made by combining the assembly of CBC-1 and CDFM21L.1. The genome assemblies were
605 annotated using the MAKER2 annotation pipeline (version 2.31.9) (76), using SNAP (version 2013–11-
606 29) (77) and Augustus (version 3.2.3) (78) as *ab initio* gene predictors. *S. cerevisiae* S288C EST and
607 protein sequences were obtained from SGD (*Saccharomyces* Genome Database,
608 http://www.yeastgenome.org/) and were aligned using BLASTX on the obtained polished sequence
609 assembly (BLAST version 2.2.28+) (79). Predicted translated protein sequences of the final gene
610 model were aligned to the *S. cerevisiae* S288C protein Swiss-Prot database using BLASTP
611 (http://www.uniprot.org/). Custom-made Perl scripts were used to map systematic names to the
612 annotated gene names (Supplementary Table 2). Error rates in nanopore-sequencing data were
613 estimated from the q score (Phred scaled) per read, as calculated by the base caller Albacore (version
614 1.1.0) (Oxford Nanopore Technology). Average q score was used to calculate the error $P= 10^{q/10}$.

615

616 **RNA isolation**

617 CDFM21L.1, IMX1765, IMX1939 and HTS020 were grown in SMG, SMM and SMMt until mid-
618 exponential phase (OD_{660nm} of 12 for SMG/SMM and of OD_{660nm} 15 for SMMt). Culture samples
619 corresponding to ca. 200 mg of biomass dry weight were directly quenched in liquid nitrogen. The
620 samples were processed and total RNA extracted as previously described (80). Prior to cDNA
621 synthesis, purity, concentration and integrity of the RNA in the samples was assessed with Nanodrop
622 (Thermo Scientific), Qubit (Thermo Scientific) and Tapestation 220 with RNA Screen Tape (Agilent
623 Technologies), respectively, according the manufacturers' recommendations. cDNA libraries were
624 prepared using the TruSeq RNA V2 kit (Illumina). Paired-end sequencing (2 X 150 bp) was performed
625 on a 300 bp PCR-free insert library on a HISeq 2500 (Illumina) at Novogene (HK) Company Ltd (Hong
626 Kong, China). Duplicate biological samples were processed, generating an average sequence quantity
627 of 23.7M reads per sample. Reads were aligned to the CDFM21L.1 reference assembly (GEO
628 (https://www.ncbi.nlm.nih.gov/geo/) accession number GSE133146) using a two-pass STAR (81)
629 procedure. In the first pass, splice junctions were assembled and used to inform the second round of
630 alignments. Introns between 15 and 4000 bp were allowed, and soft clipping was disabled to prevent
631 low-quality reads from being spuriously aligned. Ambiguously-mapped reads were removed from the
632 dataset. Expression level for each transcript were quantified using htseq-count (82) in union mode.
633 Fragments per kilo-base of feature (gene) per million reads mapped (FPKM) values were calculated
634 by "Applying the fpkm method" from the edgeR package (83, 84). Differential expression analysis was
635 performed using DESeq (85).

636

637 ## Accession Numbers

638 The sequencing data were deposited at NCBI (https://www.ncbi.nlm.nih.gov/) under the Bioproject
639 PRJNA528469 and the transcriptomics data were deposited at GEO (Genome Expression Omnibus
640 https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE133146.

641

642 ## Supplementary Data

643 Supplementary Data are available

644

645 ## Authors contributions

646 JMD conceived the study and designed the experiments. NB, AB, LvbE, ARGdV, SMW and JD
647 performed the experimental work. NB and MvdB performed bioinformatics analysis. NB, AB, FYB, JTP
648 and JMGD supervised the study and wrote the manuscript. All authors read and approved the final
649 manuscript.

650 ## Acknowledgements

651 We thank Flip de Groot for his technical assistance in gene editing in *S. eubayanus*, Niels G.A.
652 Kuijpers, Viktor M. Boer and Jan-Maarten Geertman for their critical input.

653

654 ## Funding

655 This work was supported by the BE-Basic R&D Program (http://www.be-basic.org/), which was
656 granted an FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation
657 (EL&I).

658

Conflict of Interest

The authors declare no conflict of interest.

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896

897 **Table 1: *Saccharomyces* strains used in this study.** The abbreviation *malΔ* indicates *mal11-mal12*::loxP *mal21-mal22*::loxP
898 *mal31-32*::loxP. Mal and Mtt denote the maltose and maltotriose phenotype respectively.

Name	Species	Relevant genotype	Origin
CDFM21L.1	*S. eubayanus*	Wildtype Mal$^-$ Mtt$^-$	(38)
ABFM5L.1	*S. eubayanus*	Wildtype Mal$^-$ Mtt$^-$	(38)
CBS 12357	*S. eubayanus*	Wildtype Mal$^+$ Mtt$^-$	(1) Westerdijk institute[a]
CBS 1483	*S. pastorianus*	Wildtype Mal$^+$ Mtt$^+$	Westerdijk institute[a]
CEN.PK113-7D	*S. cerevisiae*	*MATa MAL1x MAL2x MAL3x MAL4x MAL2-8C SUC2 LEU2 URA3*	(86)
IMZ616	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mp2/3hΔ suc2Δ ima1Δ* ima2Δ *ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9 URA3 ARS4 CEN6*)	(44)
IMX1365	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ::* *ScTDH3$_p$-ScMAL12-ScADH1$_t$ ScTEF1$_p$-ScAGT1-ScCYC1$_t$*	(13)
IMX1702	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ::* *ScTDH3$_p$-ScMAL12-ScADH1$_t$ ScTEF1$_p$-SeMALT1-ScCYC1$_t$*	This study
IMX1704	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ::* *ScTDH3$_p$-ScMAL12-ScADH1$_t$ ScTEF1$_p$-SeMALT2-ScCYC1$_t$*	This study
IMX1706	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ::* *ScTDH3$_p$-ScMAL12-ScADH1$_t$ ScTEF1$_p$-SeMALT3-ScCYC1$_t$*	This study
IMX 1708	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*) *sga1Δ::* *ScTDH3$_p$-ScMAL12-ScADH1$_t$ ScTEF1$_p$-SeAGT1-ScCYC1$_t$*	This study
IMX1313	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1Δ::*ScTEF1$_p$-ScMAL31-ScCYC1$_t$* pUDC156 (*URA3 cas9*)	This study
IMX1313Δ	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1Δ::*ScTEF1$_p$-ScMAL31-ScCYC1$_t$*	This study
IMZ752	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1Δ::*ScTEF1$_p$-ScMAL31-ScCYC1$_t$* pUDE843 (*ori* (*ColE1*) *bla* 2µ *ScTDH3$_p$-SeMALS1-ScADH1$_t$ URA3*)	This study
IMZ753	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mph2/3Δ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* sga1Δ::*ScTEF1$_p$-ScMAL31-ScCYC1$_t$* pUDE844 (*ori* (*ColE1*) *bla* 2µ *ScTDH3$_p$-SeMALS2-ScADH1$_t$ URA3*)	This study
IMK820	*S. eubayanus*	*MATa/MATα Semalt1Δ/Semalt1Δ*	This study
IMK823	*S. eubayanus*	*MATa/MATα Seagt1Δ/Seagt1Δ* (X3)	This study
IMX1939	*S. eubayanus*	*MATa/MATα Semalt1Δ/Semalt1Δ Sesga1Δ::ScMAL13/Sesga1Δ::ScMAL13*	This study
IMX1940	*S. eubayanus*	*MATa/MATα Seagt1Δ/Seagt1Δ Sesga1Δ::ScMAL13/Sesga1Δ::ScMAL13*	This study
IMX1762	*S. eubayanus*	*MATa/MATα Sesga1Δ::ScMAL12/Sesga1Δ::ScMAL12*	This study
IMX1765	*S. eubayanus*	*MATa/MATα Sesga1Δ::ScMAL13/Sesga1Δ::ScMAL13*	This study
CBC-1	*S. cerevisiae*	*MATa/MATα* Mal$^+$ Mtt$^-$	Lallemand
HTSH020	*S. cerevisiae X S. eubayanus*	*MATa/MATα* Mal$^+$ Mtt$^+$	This study

899 [a] Westerdijk Fungal Biodiversity Institute www.westerdijkinstitute.nl/

900

901 **Table 2 Plasmids used in this study**

Plasmid	Relevant genotype	Origin
p426-TEF-amdS	*ori* (*ColE1*) *bla* 2µ amdSYM *TEF1*$_{pr}$-*CYC1*$_{ter}$	(64)
pUD444	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*ScMAL31*-*ScCYC1*$_{ter}$	This study
pUD794	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT1*-*ScCYC1*$_{ter}$	This study
pUD795	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT2*-*ScCYC1*$_{ter}$	This study
pUD796	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeMALT3*-*ScCYC1*$_{ter}$	This study
pUD797	*ori* (*ColE1*) *bla* 2µ amdSYM *ScTEF1*$_{pr}$-*SeAGT1*-*ScCYC1*$_{ter}$	This study
pUDE044	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*ScMAL12*-*ScADH1*$_{ter}$ *URA3*	(65)
pUDE843	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*SeMALS1*-*ScADH1*$_{ter}$ *URA3*	This study
pUDE844	*ori* (*ColE1*) *bla* 2µ *ScTDH3*$_{pr}$-*SeMALS2*-*ScADH1*$_{ter}$ *URA3*	This study
pUDE780	*ori* (*ColE1*) *bla* 2µ *ScPGK1*$_{pr}$-*ScMAL13*-*ScTEF2*$_{ter}$ *URA3*	This study
pUDP002	*ori* (*ColE1*) *bla* panARSopt *Hyg ScTDH3*$_{pr}$-BsaI-BsaI-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	(68)
pUDP004	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$-BsaI-BsaI-Sc*CYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	(47)
pUDP052	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SGA1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	(13)
pUDP091	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SeMALT1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	This study
pUDP090	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SeAGT1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	This study
pUDR119	ori (ColE1) *bla* 2µ amdSYM *SNR52*$_{pr}$-gRNA$_{ScSGA1}$-*SUP4*$_{ter}$	(70)
pYTK074	ori (ColE1) *cat URA3*	(66)
pYTK082	*cat* 2µ	(66)
pYTK083	ori (ColE1) *bla*	(66)
pUD631	ori (ColE1) *bla* gRNA$_{SeMALT1}$	(13)
pUD634	ori (ColE1) *bla* gRNA$_{SeAGT1}$	This study
pUDC156	ori (ColE1) *bla ARS4 CEN6 URA3 Spcas9*	(44)

902

903

904 **Legends to Figures**

905 **Figure 1. Genome comparison between CDFM21L.1 and CBS 12357^T. (A)** Translocations in
906 CDFM21L.1 relative to CBS12357^T, (i) a paracentric inversion in the sub-telomeric region of
907 chromosome VII, involving approximately 8 kbp, (ii) a translocation of approximately 12 kbp from the
908 left sub-telomeric region of chromosome VIII to the right sub-telomeric region of chromosome VI,
909 and (iii) a reciprocal translocation between approximately 20 kbp from the right sub-telomeric region
910 of chromosome V and approximately 60 kbp from the center of chromosome XII.Red lines depicted
911 chromosomes separation. Genome syntheny is indicated with colored blocks. **(B)** Relative
912 chromosome position of gene presence differences between CDFM21L.1 (blue) and CBS 12357^T (red).
913 **(C)** Representation of the assembled CDFM21L.1 *S. eubayanus* chromosomes, the black boxes denote
914 newly added sequences. New annotated open reading frames and gene entries modified relative to
915 the CBS 12357T draft genome (13).

916

917 **Figure 2. Organization of sub-telomeric regions involving *MAL* genes and *SeAGT1* in CDFM21L.1. (A)**
918 Chromosome sections are represented as lines and red boxes denote telomeres. CDFM21L.1 genome
919 harbors three *SeMALT* genes of which *SeMALT2* and *SeMALT3* have a mutation resulting in an early
920 stop codon and truncated protein (denoted with *). Three copies of *SeAGT1* were found close to the
921 telomeres on CHRVII, XIV and XV. Furthermore there are two intact *SeMALS* genes on CHRII (1) and
922 XII (2) and three *SeMALR* genes on CHRV (1) and XIII (2) whose copy on CHRV is also mutated
923 (*SeMALR1**). The gene and interval sizes are approximately to scale. (**B**) Phylogeny of *Saccharomyces*
924 *SeAGT1* genes described in *S. cerevisiae*, *S. eubayanus*, and lager-brewing hybrid *S. pastorianus*. (C)
925 Nucleotide percentage identities between *AGT1* orthologs from *S. cerevisiae*, *S. eubayanus*, and
926 lager-brewing hybrid *S. pastorianus*. Green color indicates highest similarity between SeAGT1 and
927 SeAGT1 genes from *S. pastorianus* strains CBS 1483 and WS3470. Red color indicates similarity
928 between SeAGT1 from North American strains with SeAGT1 genes from Asian *S. eubayanus* and *S.*
929 *pastorianus* strains CBS 1483 and WS3470.

930

931 **Figure 3. Characterization of sugar consumption of *S. pastorianus* CBS1483 (black) and *S.***
932 ***eubayanus* CBS 12357^T (blue), CDFM21L.1 (red), and ABFM5L.1 (green) on wort.** For every sample,
933 glucose (■), maltose (▲), maltotriose (▼) and ethanol (◆) were measured from the supernatant.
934 Strains were grown at 12 °C for 110 hours in infusion Neubor flasks. Samples were filtered through a
935 0.22 µm filter and analyzed on HPLC. Data represents average and standard deviation of three
936 biological replicates.

937

938 **Figure 4. Overexpression of *SeMALT*, *SeAGT1* and *SeMALS* genes in a maltose negative background**
939 ***S. cerevisiae* strain.** Maltose negative background strain IMZ616 (■), IMX1365 overexpressing
940 *ScMAL11* (▲), IMX1702 overexpressing *SeMALT1* (▼), IMX1704 overexpressing *SeMALT2* (◆)
941 IMX1706 overexpressing *SeMALT3* (●) and IMX1708 overexpressing SeAGT1 (□) were grown on SM
942 2% maltose or maltotriose at 20 °C. Growth on maltose **(A)** and on maltotriose **(B)** was monitored
943 based on optical density (OD_{660nm}) and concentrations of maltose and maltotriose in culture
944 supernatants were measured by HPLC. Data are presented as average and standard deviation of two
945 biological replicates. **(C)** IMX1313 overexpressing only *ScMAL31* (○), IMZ752 overexpressing
946 *ScMAL31* and *SeMALS1* (△) and IMZ753 overexpressing *ScMAL31* and *SeMALS2* (▽) grown on SM
947 maltose 2%. Growth was monitored based on optical density measurement at 660 nm (OD_{660nm}) and
948 maltose in culture supernatants was measured by HPLC. Data represents average and standard
949 deviation of two biological replicates.

950 **Figure 5. Integration of *ScMAL13* in CDFM21L.1 restores growth on maltose and maltotriose and**
951 **enables native *SeMALT1* and *SeAGT1* characterization in knockout strains IMK820 and IMK823. (A)**
952 Characterization of *S. eubayanus* IMX1765 overexpressing *ScMAL13* (▼) and CDFM21L.1 (■) on SM
953 with glucose, maltose or maltotriose at 20 °C. OD_{660nm} was measured (black) and sugar (black) and
954 ethanol (red) concentrations were determined from the supernatant by HPLC. **(B)** Overview of

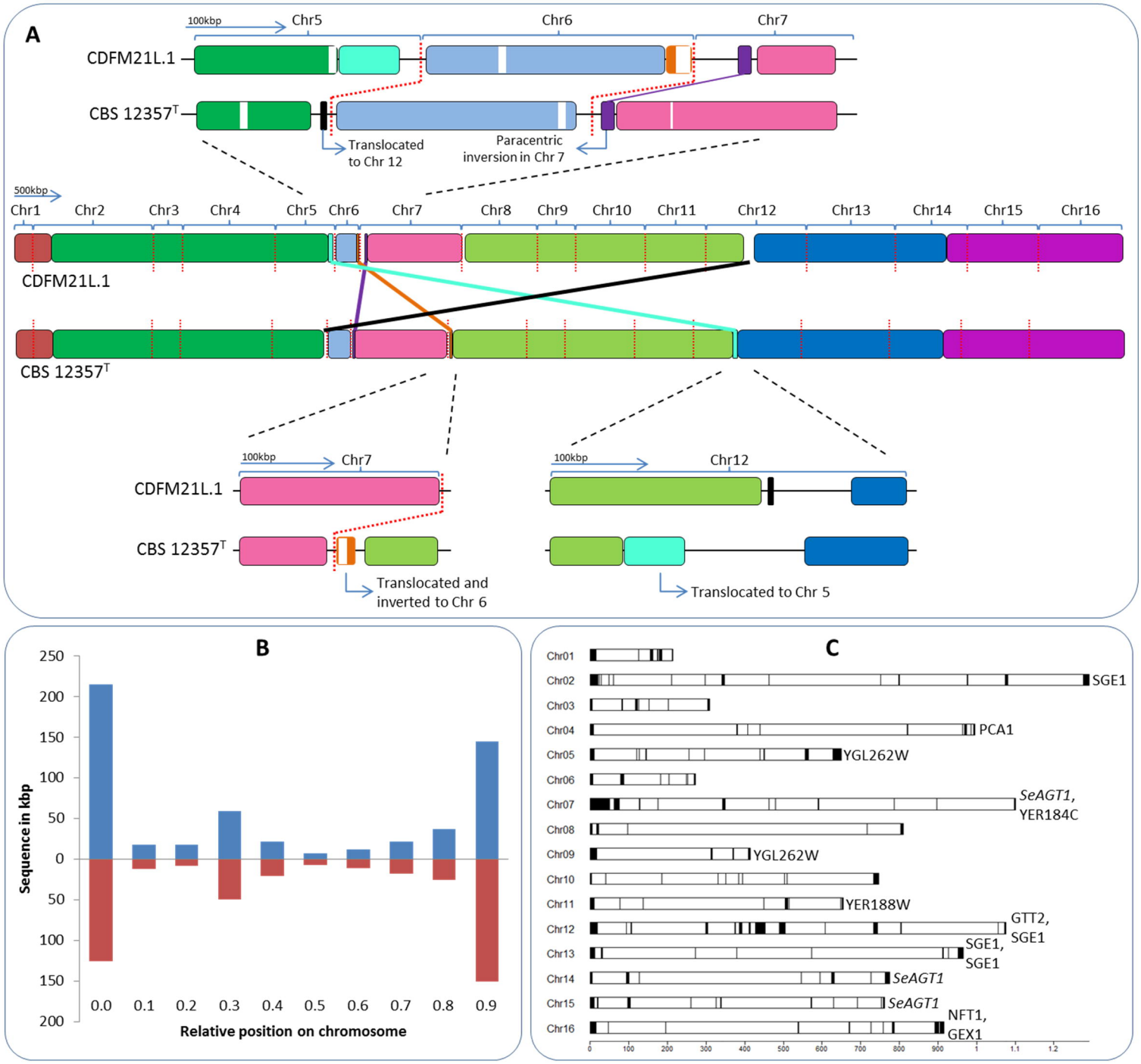
955 constructed knockout strains. Knockouts of *SeMALT1* (IMK820) and *SeAGT1* (IMK823) were made
956 with CRISPR-Cas9. Subsequently the *SeSGA1* locus was replaced by *ScPGK1*$_p$- *ScMAL13-ScTEF2*$_t$ using
957 CRISPR-Cas9 in both strains resulting in IMX1939 and IMX1940, respectively. **(C)** *S. eubayanus* strains
958 IMK820 (■), IMK823 (▲), IMX1939 (▼) and IMX1940 (◆) were characterized on SM with maltose or
959 maltotriose at 20 °C. OD_{660nm} was measured (black) and sugar (black) and ethanol (red)
960 concentrations were determined from the supernatant by HPLC. All data represents average and
961 standard deviation of biological duplicates.

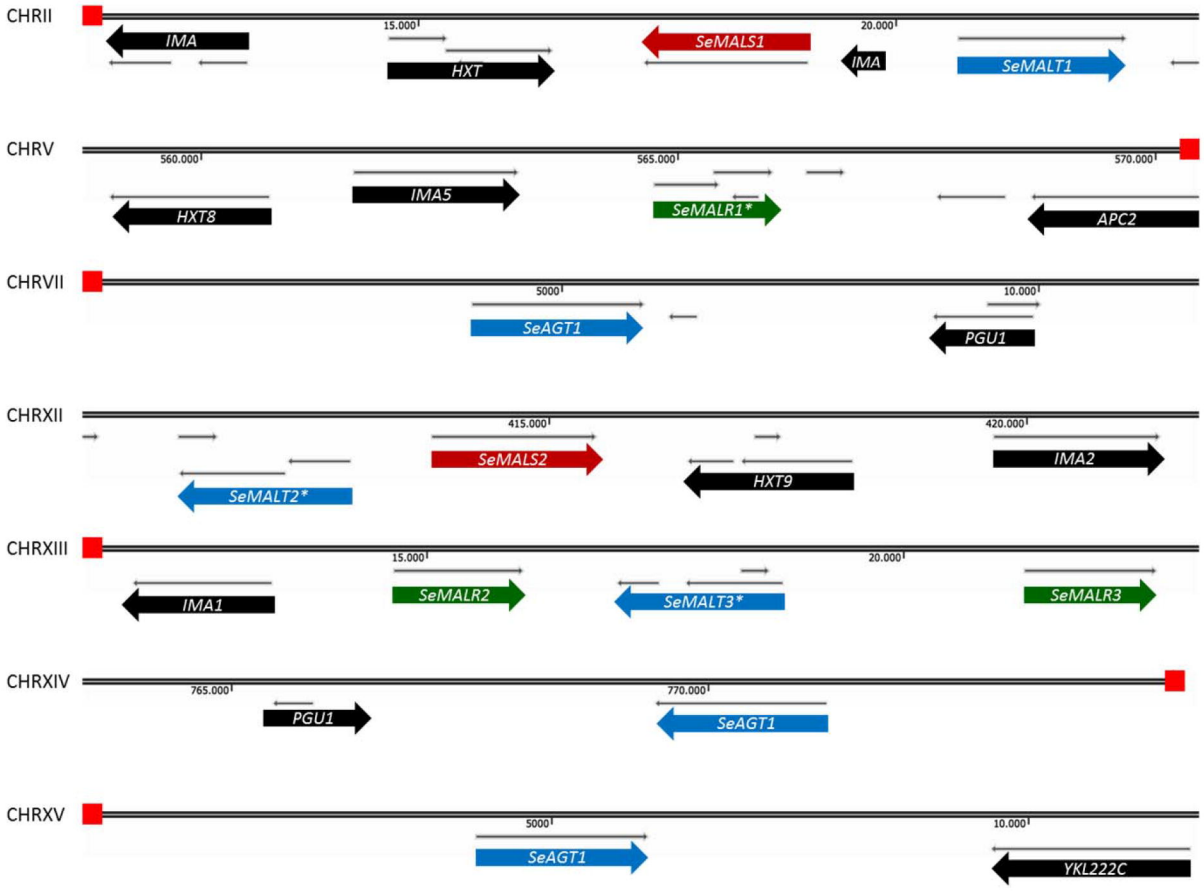
962

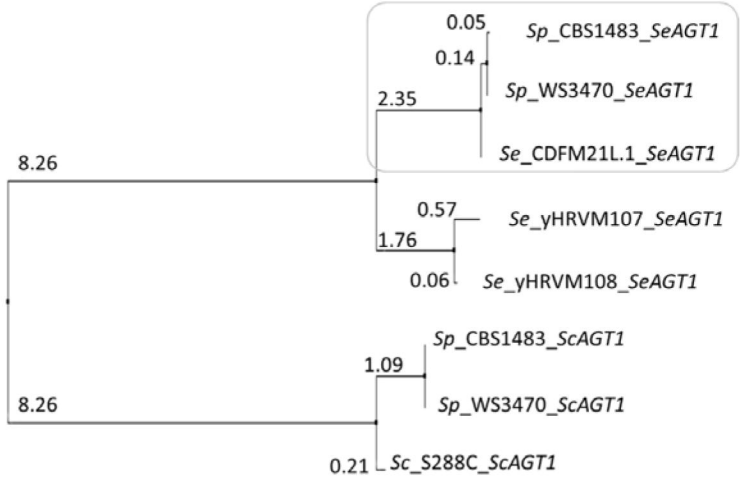
963 **Figure 6. Expression levels of maltose metabolism genes in IMX1765.** Normalized transcript levels of
964 maltose metabolism genes from IMX1765mid-exponential phase grown on glucose (red), maltose
965 (blue)and maltotriose (green) at 20 °C were calculated from duplicate RNA sequencing experiments
966 (2x 150 bp) using the FPKM method . All data represents average and standard deviation of two
967 biological duplicates.

968

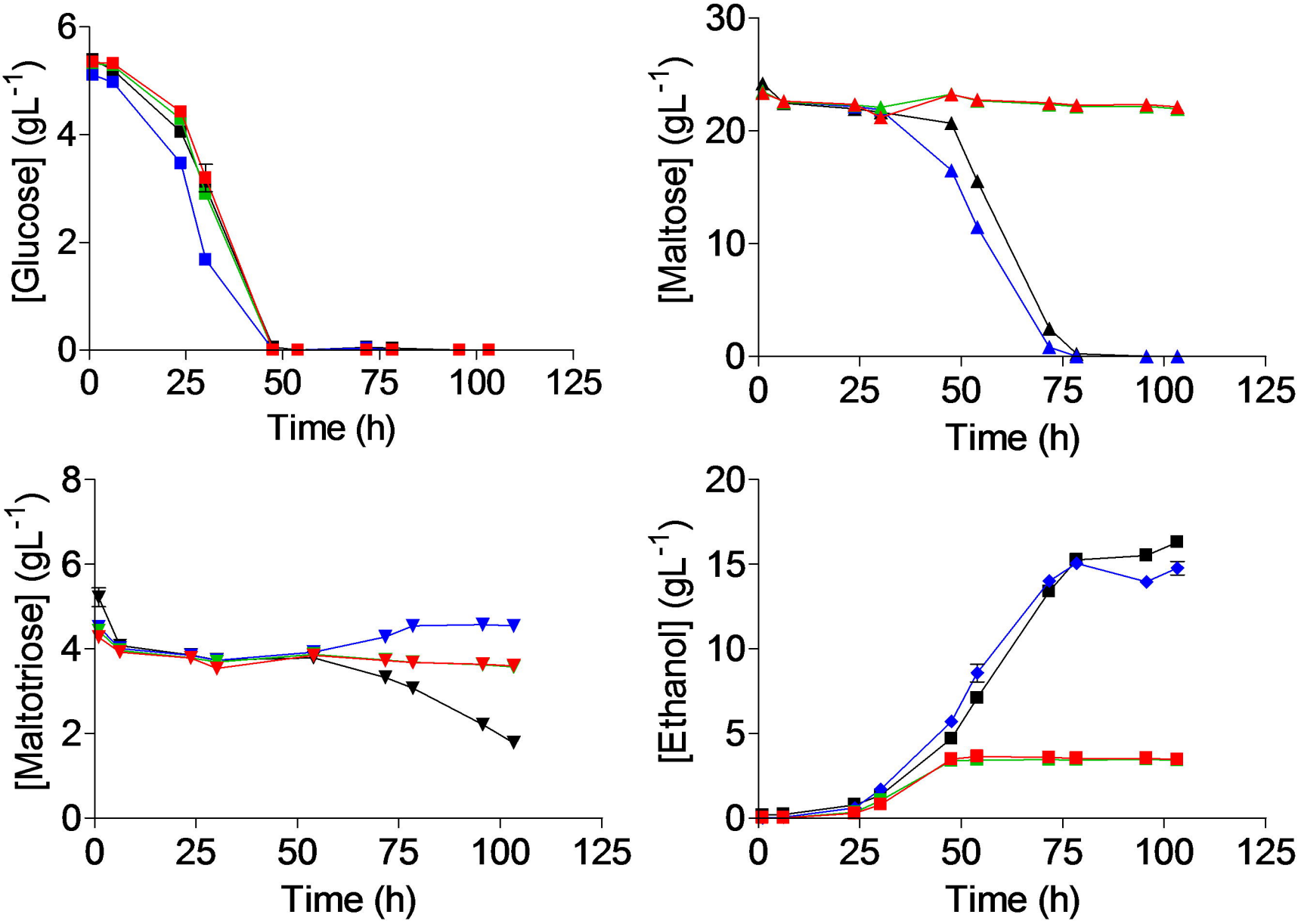
969 **Figure 7. Hybridization of maltotriose deficient *S. cerevisiae* and *S. eubayanus* leading to crosstalk**
970 **restoring maltotriose utilization, explains *S. pastorianus* phenotype. (A)** Characterization of *S.*
971 *cerevisiae* CBC-1 (▼), *S.* eubayanus CDFM21L.1 (▲) and hybrid HTS020 (■) on mock wort at 12 °C.
972 Consumption of maltose (red), maltotriose (blue) and production of ethanol (green) was measured
973 from supernatant by HPLC. Data represents average and standard deviation from biological
974 triplicates. **(B)** Normalized transcript levels of maltose metabolism genes from HTS020 mid-
975 exponential phase grown on glucose (red), maltose (blue)and maltotriose (green) at 20 °C were
976 calculated from duplicate RNA sequencing experiments (2x 150 bp) using the FPKM method . All
977 data represents average and standard deviation of two biological duplicates.


A
100kbp
Chr5
Chr6
Chr7
CDFM21L.1
CBS 12357T
Translocated to Chr 12
Paracentric inversion in Chr 7
500kbp
Chr1
Chr2
Chr3
Chr4
Chr5
Chr6
Chr7
Chr8
Chr9
Chr10
Chr11
Chr12
Chr13
Chr14
Chr15
Chr16
CDFM21L.1
CBS 12357T
100kbp
Chr7
CDFM21L.1
CBS 12357T
Translocated and inverted to Chr 6
100kbp
Chr12
Translocated to Chr 5
B
Sequence in kbp
250
200
150
100
50
0
50
100
150
200
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Relative position on chromosome
C
Chr01
Chr02
SGE1
Chr03
Chr04
PCA1
Chr05
YGL262W
Chr06
Chr07
SeAGT1, YER184C
Chr08
Chr09
YGL262W
Chr10
Chr11
YER188W
Chr12
GTT2, SGE1
Chr13
SGE1, SGE1
Chr14
SeAGT1
Chr15
SeAGT1
Chr16
NFT1, GEX1
0
100
200
300
400
500
600
700
800
900
1
1.1
1.2





	Sc_S288C_ScAGT1	Sp_WS3470_ScAGT1	Sp_CBS1483_ScAGT1	Se_CDFM21L.1_SeAGT1	Sp_WS3470_SeAGT1	Sp_CBS1483_SeAGT1	Se_yHRVM107_SeAGT1	Se_yHRVM108_SeAGT1
Sc_S288C_ScAGT1	100	98.60	100	79.94	79.88	79.03	80.19	80.59
Sp_WS3470_ScAGT1		100	100	79.21	79.16	78.32	79.34	79.75
Sp_CBS1483_ScAGT1			100	79.21	79.16	78.32	79.34	79.75
Se_CDFM21L.1_SeAGT1				100	99.89	99.84	95.10	95.64
Sp_WS3470_SeAGT1					100	99.95	94.99	95.53
Sp_CBS1483_SeAGT1						100	94.99	95.53
Se_yHRVM107_SeAGT1							100	99.35
Se_yHRVM108_SeAGT1								100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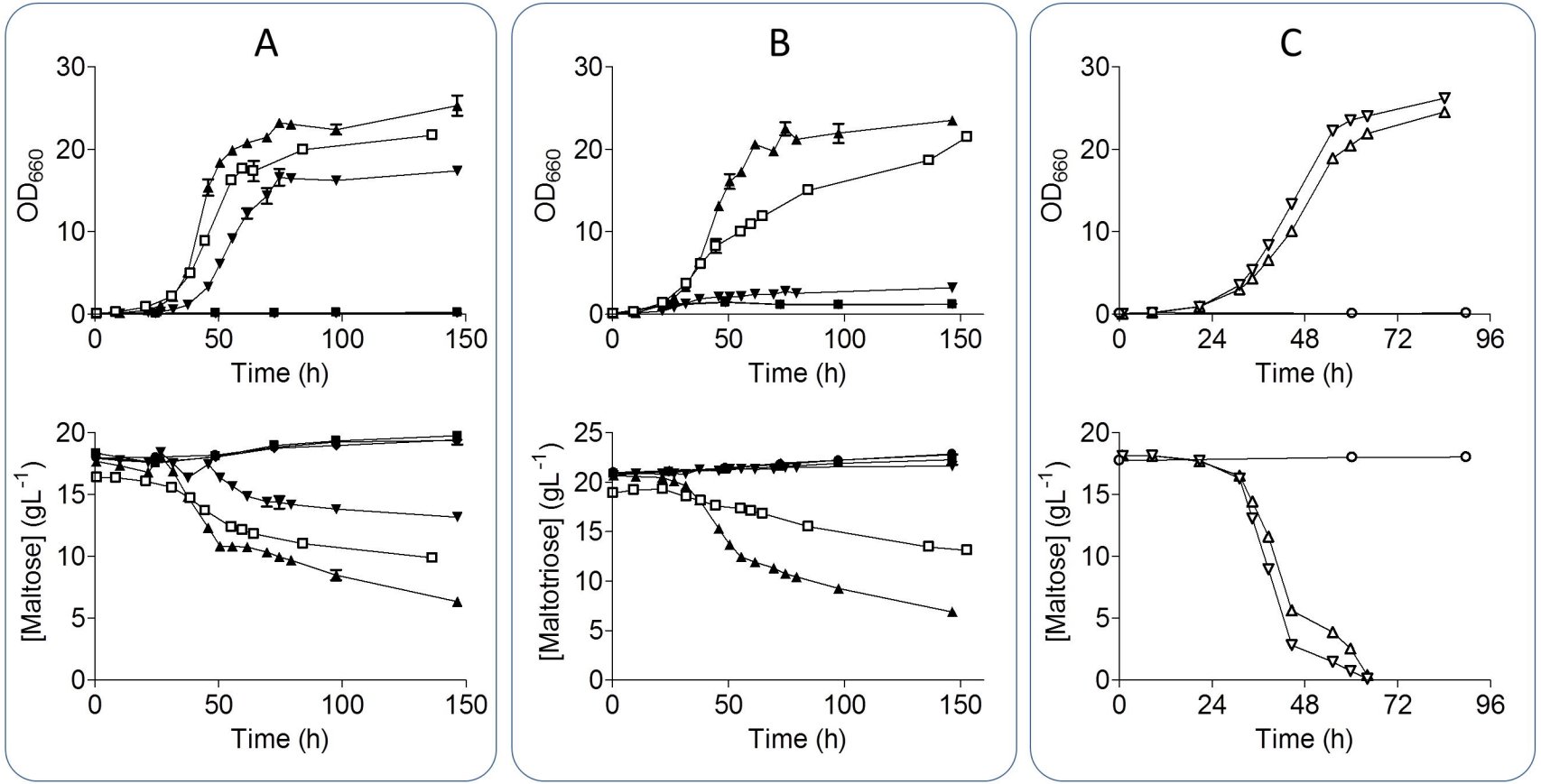
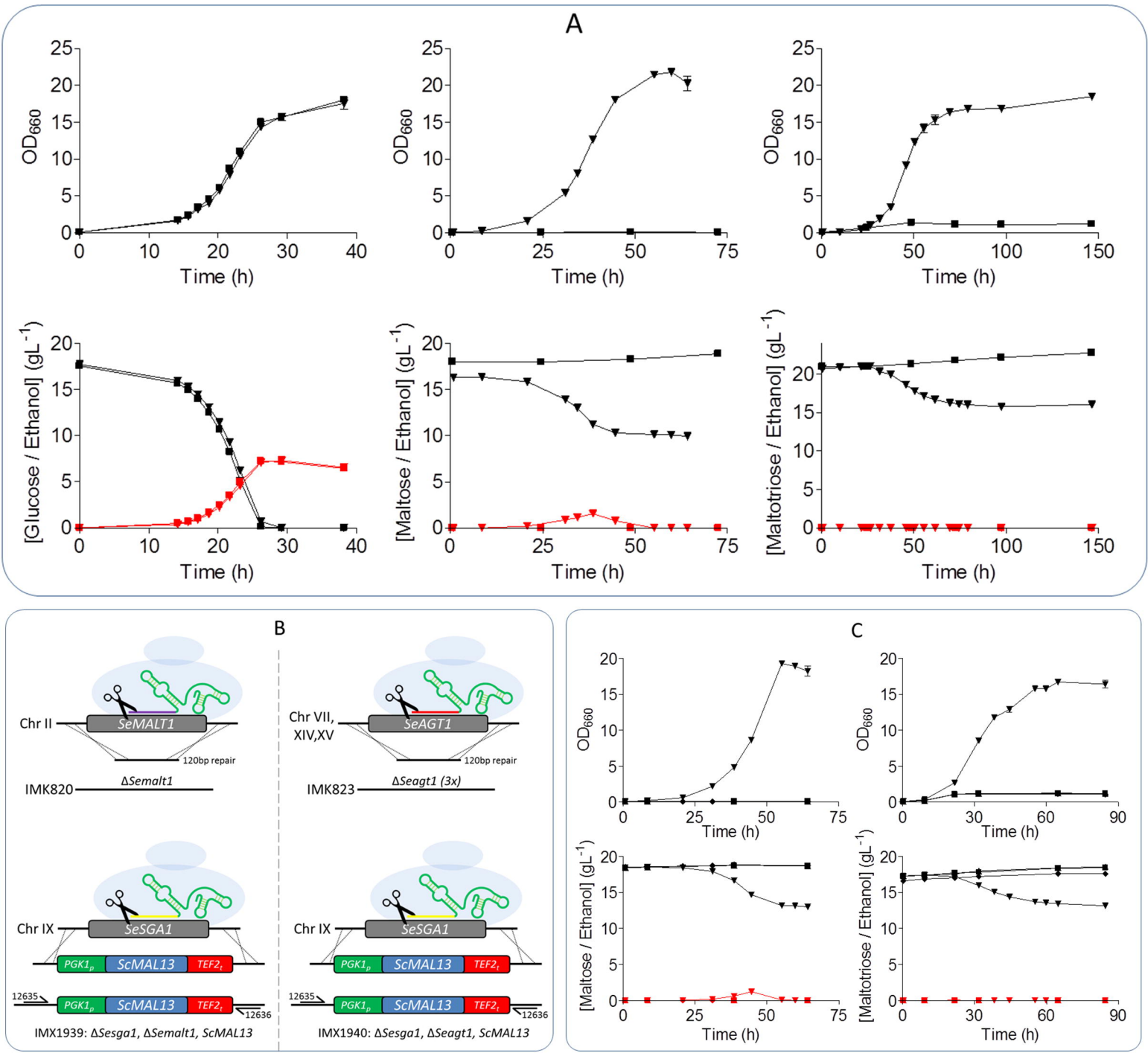
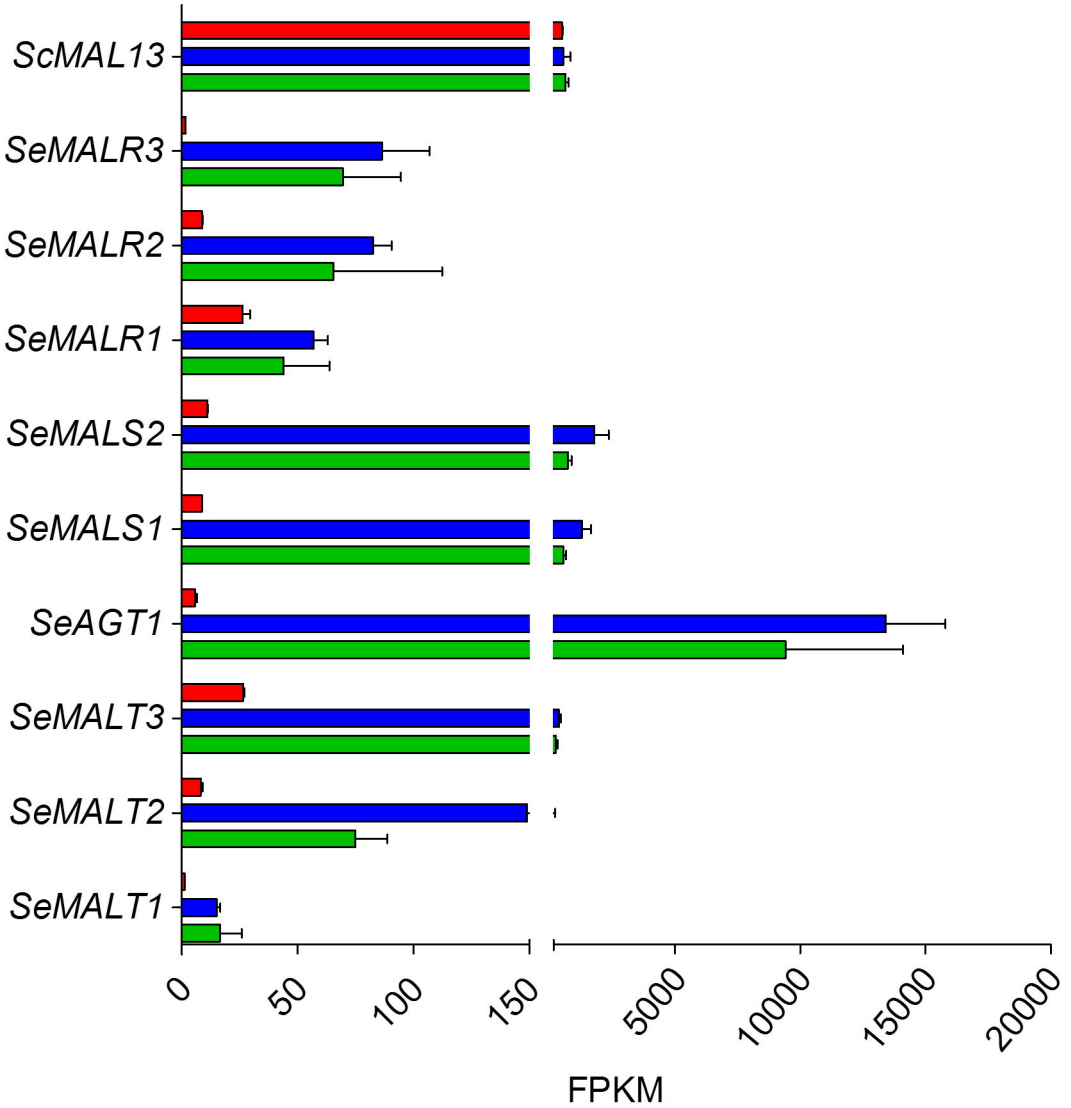
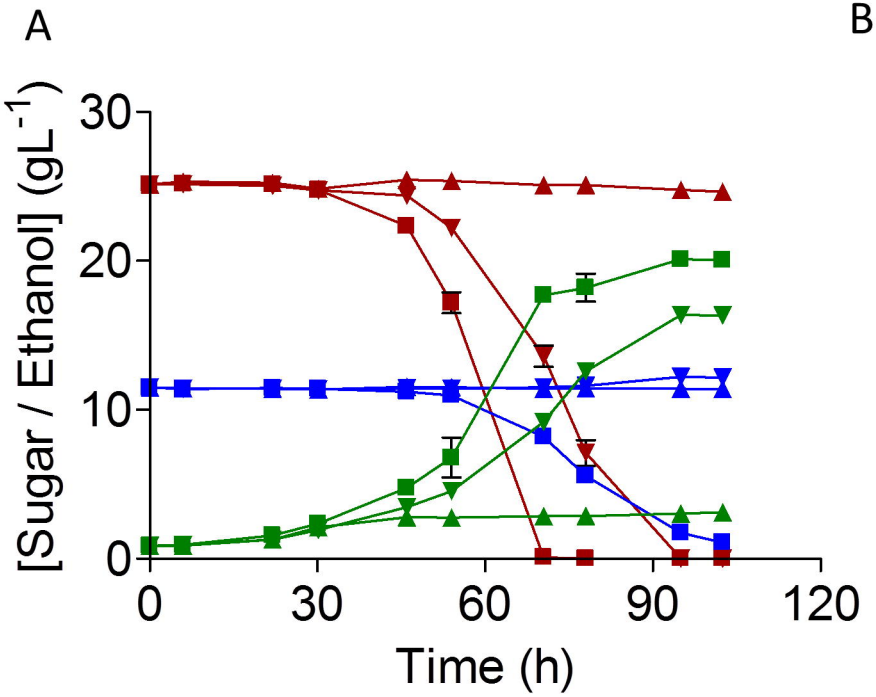
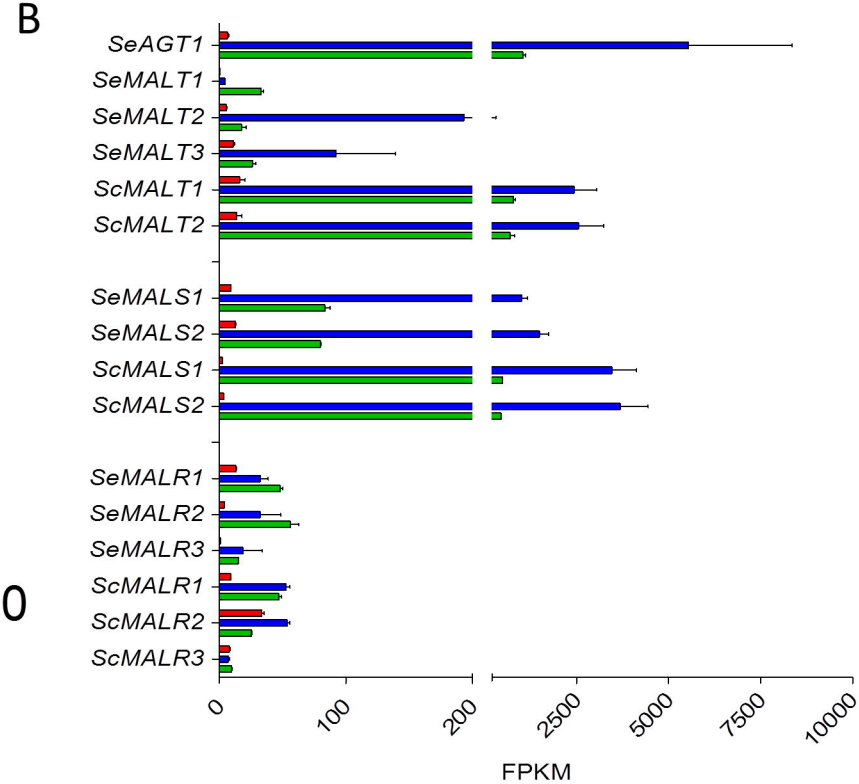
Time (h)
[Maltose] (gL-1)
Time (h)
[Maltotriose] (gL-1)
Time (h)
[Ethanol] (gL-1)
Time (h)


A
B
C
OD660
Time (h)
[Maltose] (gL-1)
[Maltotriose] (gL-1)
0
10
20
30
50
100
150
24
48
72
96
5
15
25


A
OD660
Time (h)
[Glucose / Ethanol] (gL-1)
[Maltose / Ethanol] (gL-1)
[Maltotriose / Ethanol] (gL-1)
B
Chr II
SeMALT1
120bp repair
IMK820
ΔSemalt1
Chr VII, XIV,XV
SeAGT1
IMK823
ΔSeagt1 (3x)
Chr IX
SeSGA1
PGK1p
ScMAL13
TEF2t
12635
12636
IMX1939: ΔSesga1, ΔSemalt1, ScMAL13
IMX1940: ΔSesga1, ΔSeagt1, ScMAL13
C


ScMAL13
SeMALR3
SeMALR2
SeMALR1
SeMALS2
SeMALS1
SeAGT1
SeMALT3
SeMALT2
SeMALT1
0
50
100
150
5000
10000
15000
20000
FPKM


A
[Sugar / Ethanol] (gL-1)
30
20
10
0
0
30
60
90
120
Time (h)


B
SeAGT1
SeMALT1
SeMALT2
SeMALT3
ScMALT1
ScMALT2
SeMALS1
SeMALS2
ScMALS1
ScMALS2
SeMALR1
SeMALR2
SeMALR3
ScMALR1
ScMALR2
ScMALR3
0
100
200
2500
5000
7500
10000
FPKM