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

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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.

53 ## Introduction

54 *Saccharomyces pastorianus* is an interspecific hybrid of *S. cerevisiae* and *S. eubayanus* (Libkind et al.
55 2011; Nakao et al. 2009; Walther et al. 2014; Van den Broek et al. 2015). *S. pastorianus* strains are
56 widely used for production of lager beer, which is currently the most produced alcoholic beverage
57 worldwide. Lager brewing requires alcoholic fermentation at relatively low temperatures. *S.*
58 *pastorianus* was hypothesized to have emerged by spontaneous hybridization and to have colonized
59 early lager-brewing processes due to a combination of cold-tolerance inherited from *S. eubayanus*
60 and superior fermentation kinetics inherited from *S. cerevisiae* (Krogerus et al. 2015a; Hebly et al.
61 2015; Krogerus et al. 2017a). Lager beer is brewed from barley wort, whose sugar composition
62 consists, by weight, of approximately 15% glucose, 60% maltose and 25% maltotriose (Zastrow et al.
63 2001). During wort fermentation, maltotriose is generally only utilized after glucose and maltose are
64 depleted, while its consumption is also relatively slow and often incomplete (Alves et al. 2008;
65 Vidgren et al. 2009; Brickwedde et al. 2017).

66 Complete sugar utilization is desirable for lager beer fermentation to optimize concentrations of
67 ethanol and flavor compounds and to avoid residual sweetness (Zheng et al. 1994). While *S.*
68 *pastorianus* and *S. cerevisiae* strains are capable of consuming maltotriose, none of the wild isolates
69 of *S. eubayanus* characterized thus far have been shown to possess this trait (Hebly et al. 2015;
70 Brickwedde et al. 2018; Baker and Hittinger 2018). These observations led to the hypothesis that the
71 ability of *S. pastorianus* to ferment maltotriose was inherited from *S. cerevisiae* (Brickwedde et al.
72 2018; Hebly et al. 2015; Krogerus et al. 2017b, 2015b; Nikulin et al. 2018).

73 The genetic information for maltose utilization is well conserved in *Saccharomyces* species and
74 depends on three gene families. *MALT* genes encode plasma-membrane proton symporters with
75 varying substrate specificities and affinities (Cheng and Michels 1991; Vidgren et al. 2010), *MALS*
76 genes encode α-glucosidases that hydrolyze α-oligoglucosides into glucose, while *MALR* genes
77 encode a regulator required for transcriptional induction of *MALT* and *MALS* genes by maltose

78 (Naumov et al. 1994; Charron et al. 1989). In *Saccharomyces* species, maltose-utilization genes are
79 generally organized in *MAL* loci. These loci contain a *MALT* gene (called *ScMALx1* and *SeMALTx* in *S.*
80 *cerevisiae* and *S. eubayanus*, respectively), a *MALS* gene referred to as *ScMALx2* or *SeMALSx* and a
81 *MALR* gene referred to as *ScMALx3* or *SeMALRx* (Hayford and Jespersen 1999; Brickwedde et al.
82 2018). In the absence of glucose and presence of maltose, the MalR regulator binds a bi-directional
83 promoter, thereby simultaneously activating expression of *MALT* and *MALS* genes (Bell et al. 1997).

84 The Sc*MAL1*-Sc*MAL4* and Sc*MAL6* loci of *S. cerevisiae* as well as the *SeMAL1-SeMAL4* loci of *S.*
85 *eubayanus* are located in subtelomeric regions (Barnett 1976; Salazar et al. 2017; Brickwedde et al.
86 2018; Baker et al. 2015). While all *S. cerevisiae Sc*Malx1 transporters transport maltose, only *Sc*Mal11
87 is able to also transport maltotriose (Alves et al. 2008). *ScMAL11* (also known as *ScAGT1*) shares only
88 57% nucleotide identity with other *ScMALx1* genes (Han et al. 1995). The four *SeMALT* (*SeMALT1-4*)
89 genes identified in the genome of the Patagonian type strain FM1318/CBS 12357 of *S. eubayanus*
90 were shown to encode functional maltose transporters, but none of these genes enabled maltotriose
91 transport (Brickwedde et al. 2018). While no clear *ScAGT1* ortholog was found in *S. eubayanus* CBS
92 12357^{T}, such an ortholog was recently found in the genomes of two North American isolates assigned
93 to the Holarctic subclade of *S. eubayanus* (Baker and Hittinger 2018).

94 *S. pastorianus* inherited *MAL* genes from both *S. cerevisiae* and *S. eubayanus* (Van den Broek et al.
95 2015; Nakao et al. 2009; Vidgren and Gibson 2018). However, the *S. cerevisiae*-derived maltotriose-
96 transporter gene *ScAGT1* is truncated and, therefore, non-functional in *S. pastorianus* (Vidgren et al.
97 2009). Instead, maltotriose consumption by *S. pastorianus* strains was attributed to *SeAGT1* and
98 *SpMTY1*/*SpMTT1* genes (Salema-Oom et al. 2005; Dietvorst et al. 2005; Cousseau et al. 2013; Nguyen
99 et al. 2011). In *S. pastorianus*, *SeAGT1* is located on *S. eubayanus* CHRXV and was therefore, already
100 before the identification of an *AGT1* ortholog in Holarctic *S. eubayanus* strains (Baker and Hittinger
101 2018), assumed to originate from *S. eubayanus* (Nakao et al. 2009). *SpMTY1,* also referred to as
102 *SpMTT1,* is located on *S. cerevisiae* CHRVII and has less than 92% sequence identity with other

103 *Saccharomyces* maltose transporters (Dietvorst et al. 2005). However, *SpMTY1* contains sequence
104 patches with high similarity to maltose transporters from *S. eubayanus* and *S. paradoxus* (Brouwers
105 et al. 2018). Recently, two independent laboratory evolution studies with *S. eubayanus*
106 demonstrated that recombination of different *SeMALT* genes yielded chimeric, neo-functionalized
107 genes that encoded maltotriose transporters (Brouwers et al. 2019; Baker and Hittinger 2019).
108 *SpMTY1* may have resulted from successive introgressions of maltose-transporter genes from *S.*
109 *cerevisiae*, *S. eubayanus* and *S. paradoxus*.

110 Recently made *S. cerevisiae* x *S. eubayanus* laboratory hybrids showed similar lager-brewing
111 performance as *S. pastorianus* strains, also with respect to maltotriose utilization (Hebly et al. 2015;
112 Mertens et al. 2015; Krogerus et al. 2015b; Nikulin et al. 2018). In these hybrids, maltotriose
113 consumption depended on the presence of a functional *Sc*Agt1 transporter encoded by the *S.*
114 *cerevisiae* subgenome (Gorter de Vries et al. 2018). However, in view of the non-functionality of
115 *ScAGT1* in current *S. pastorianus* strains, these laboratory hybrids did not fully recapitulate the
116 genetic landscape of *S. pastorianus* with respect to maltotriose fermentation (Hebly et al. 2015;
117 Mertens et al. 2015; Nakao et al. 2009).

118 Studies on laboratory hybrids based on *S. eubayanus* strains whose genomes are more closely related
119 to the *S. eubayanus* subgenome of *S. pastorianus* strains than that of the Patagonian type strain
120 CBS12357 might generate new insights into the evolution of maltotriose utilization in *S. pastorianus*.
121 To date, Himalayan *S. eubayanus* isolates show the highest sequence identity with the *S. eubayanus*
122 sub-genome of *S. pastorianus*, with up to 99.82% identity, as opposed to 99.56% for *S. eubayanus*
123 CBS12357^{T} (Bing et al. 2014).

124 Here, we investigated if and how the genomes of Himalayan *S. eubayanus* strains could have
125 contributed to maltotriose utilization in the earliest hybrid ancestors of current *S. pastorianus*
126 strains. To this end, we generated chromosome-level genome assemblies of these strains by long-
127 read DNA sequencing. Since the Himalayan strains were unable to utilize maltotriose, we functionally

128 characterized the assembled *MAL* genes and identified genetic determinants that prevented
129 maltotriose utilization. Subsequently, a laboratory hybrid of a representative Himalayan *S. eubayanus*
130 strain and a maltotriose-deficient ale strain of *S. cerevisiae* was generated to investigate the genetics
131 of maltotriose utilization in a hybrid context. We discuss the implications of the experimental results
132 for the proposed role and origin of *SeAGT1* in *S. pastorianus* and for the potential of hybridization to
133 enable maltotriose consumption in novel *Saccharomyces* hybrids.

134 ## Materials and methods

135 **Strains and maintenance**

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

140 **Media and cultivation**

141 *S. eubayanus* batch cultures were grown on synthetic medium (SM) containing 3.0 g L^{-1} KH_2PO_4, 5.0 g
142 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
143 (Verduyn et al. 1992). The pH was set to 6.0 with 2 M KOH prior to autoclaving at 120 °C for 20 min.
144 Vitamin solutions were sterilized by filtration and added to the sterile medium. Concentrated sugar
145 solutions were autoclaved at 110 °C for 20 min or filter sterilized and added to the sterile flasks to
146 give a final concentration of 20 g L^{-1} glucose (SMG), maltose (SMM) or maltotriose (SMMt). With the
147 exception of IMZ752 and IMZ753, *S. cerevisiae* batch cultures were grown on SM supplemented with
148 150 mg L^{-1} uracil (Pronk 2002) to compensate for loss of plasmid pUDC156 that carried the *Spcas9*
149 endonuclease gene, and supplemented with 20 g L^{-1} glucose (SM_uG), maltose (SM_uM) or maltotriose
150 (SM_uMt). All batch cultures were grown in 250 mL shake flasks with a working volume of 50 mL. The
151 cultures were inoculated at an initial OD_{660nm} of 0.1 and incubated under an air atmosphere and
152 shaken at 200 rpm and at 20 °C in a New Brunswick™ Innova44 incubator (Eppendorf Nederland B.V,
153 Nijmegen, The Netherlands).

154 *S. eubayanus* strains transformed with plasmids pUDP052 ($gRNA_{SeSGA1}$), pUDP091 ($gRNA_{SeMALT1}$) and
155 pUDP090 ($gRNA_{SeAGT1}$) were selected on modified SMG medium ($SM_{Ace}G$) in which $(NH_4)_2SO_4$ was
156 replaced by 6.6 g L^{-1} K_2SO_4 and 10 mM acetamide (Solis-Escalante et al. 2013). SM-based solid media
157 contained 2 % Bacto Agar (BD Biosciences, Franklin Lakes, NJ). *S. cerevisiae* strains expressing either
158 *SeMALT, SeMALS* or *ScMALR* were selected on $SM_{Ace}G$. For plasmid propagation, *E. coli* XL1-Blue-
159 derived strains (Agilent Technologies, Santa Clara, CA) were grown in Lysogeny Broth medium (LB, 10

160 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) supplied with 100 mg L^{-1} ampicillin. Synthetic wort
161 medium (SWM) for growth studies contained 14.4 g·L^{-1} glucose, 2.3 g·L^{-1} fructose, 85.9 g·L^{-1} maltose,
162 26.8 g·L^{-1} 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
163 solution, 1 mL·L^{-1} vitamin solution, supplemented with the anaerobic growth factors ergosterol and
164 Tween 80 (0.01 g·L^{-1} and 0.42 g·L^{-1} respectively), as previously described (Verduyn et al. 1992).
165 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}
166 fructose and 269 mg L^{-1} FAN) was provided by Heineken Supply Chain B.V. (Zoeterwoude, the
167 Netherlands). The wort was supplemented with 1.5 mg L^{-1} of Zn^{2+} by addition of $ZnSO_4.7H_2O$,
168 autoclaved for 30 min at 121 °C, filtered using Nalgene 0.2 µm SFCA bottle top filters (Thermo
169 Scientific) and diluted with sterile demineralized water. Sporulation medium consisted of 2 % (w/v)
170 KAc in MilliQ water set to pH 7.0 with KOH, autoclaved at 121 °C for 20 min.

171 **Microaerobic growth experiments**

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

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

187 **Analytical methods**

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

196 **Plasmid construction**

197 Plasmids used and constructed in this study are listed in Table 2, oligonucleotide primers used in this
198 study are listed in Supplementary Table 1. Coding regions of *SeMALT1*, *SeMALT2*, *SeMALT3* and
199 *SeAGT1* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity DNA polymerase
200 (Thermo Scientific), according to the supplier's instructions with primers pairs 12355/12356,
201 12357/12358, 12359/12360 and 12361/12362, respectively. The coding sequence of *ScMAL31* was
202 amplified from CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo
203 Scientific), according to the supplier's instructions with primer pairs 9942/9943. Each primer carried a
204 40 bp extension complementary to the plasmid backbone of p426-TEF-amdS (Marques et al. 2017),
205 which was PCR amplified using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and
206 primers 7812/5921. Each transporter fragment was assembled with the p426-TEF-amdS backbone
207 fragment using NEBuilder HiFi DNA Assembly (New England Biolabs, Ipswich, MA), resulting in
208 plasmids pUD444 (*ScMAL31*), pUD794 (*SeMALT1*), pUD795 (*SeMALT2*), pUD796 (*SeMALT3*) and

209 pUD797 (*SeAGT1*). All plasmids were verified for correct assembly by Sanger sequencing (Baseclear,
210 Leiden, The Netherlands).

211 *SeMALS1* and *SeMALS2* were amplified from CDFM21L.1 genomic DNA with Phusion High-Fidelity
212 DNA polymerase (Thermo Scientific), with primers pairs 14451/14453 and 14452/14453,
213 respectively. Each primer pair carried a 30 bp extension complimentary to the plasmid backbone of
214 pUDE044 (de Kok et al. 2011) which was PCR amplified using Phusion High-Fidelity DNA polymerase
215 (Thermo Scientific) and primers 14449/14450. Resulting amplicons were assembled using NEBuilder
216 HiFi DNA Assembly (New England Biolabs), resulting in plasmids pUDE843 (*SeMALS1*) and pUDE844
217 (*SeMALS2*) that were verified by Sanger sequencing (Baseclear).

218 *S. cerevisiae ScMAL13*, the *ScPGK1* promoter and the *ScTEF2* terminator were amplified from
219 CEN.PK113-7D genomic DNA with Phusion High-Fidelity DNA polymerase (Thermo Scientific), with
220 primer pairs 12915/12916, 9421/9422 and 10884/10885, respectively. Fragments were gel purified
221 and used with pYTK074, pYTK082 and pYTK083 in Golden Gate assembly according to the yeast
222 toolkit protocol (Lee et al. 2015) resulting in pUDE780, which was verified by Sanger sequencing
223 (Baseclear).

224 Guide-RNA (gRNA) sequences for deletion of *SeMALT1* and *SeAGT1* in CDFM21L.1 were designed as
225 described previously (Gorter de Vries et al. 2017). The DNA sequences encoding these gRNAs were
226 synthesized at GeneArt (Thermo Scientific) and were delivered in pUD631 and pUD634, respectively.
227 The gRNA spacer sequences (Se*MALT1* 5' CCCCGATATTCTTTACACTA 3', Se*AGT1* 5'-
228 AGCTTTGCGAAAATATCCAA -3') and the structural gRNA sequence were flanked at their 5' ends by
229 the Hammerhead ribozyme (HH) and at their 3' ends by the Hepatitis Delta Virus ribozyme (HDV)
230 (Gao and Zhao 2014). The HH-gRNA-HDV fragment was flanked on both ends with a BsaI site for
231 further cloning (Juergens et al. 2018; Gorter de Vries et al. 2017). Plasmids pUDP091 ($gRNA_{SeMALT1}$)
232 and pUDP090 ($gRNA_{SeAGT1}$) were constructed by Golden Gate cloning by digesting pUDP004 and the
233 gRNA-carrying plasmid (pUD631 and pUD634, respectively) using BsaI and ligating with T4 ligase

234 (Engler et al. 2008). Correct assembly was verified by restriction analysis with PdmI (Thermo
235 Scientific) and Sanger sequencing (Baseclear).

236 **Strain construction**

237 *S. cerevisiae* IMZ616, which cannot grow on α-glucosides (Marques et al. 2018), was used as a host to
238 test functionality of individual *S. eubayanus* (putative) maltose transporter genes (Brickwedde et al.
239 2018). *S. cerevisiae* IMX1702 was constructed by integrating *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ and *ScTEF1*$_p$-
240 *SeMALT1-ScCYC1*$_t$ at the *ScSGA1* locus of strain IMZ616. A fragment containing the *ScTDH3*$_p$-
241 *ScMAL12-ScADH1*$_t$ transcriptional unit was PCR amplified using Phusion High-Fidelity DNA
242 polymerase (Thermo Scientific) from pUDE044 with primers 9596/9355, which included a 5'
243 extension homologous to the upstream region of the *ScSGA1* locus and an extension homologous to
244 the co-transformed transporter fragment, respectively. The DNA fragment carrying the *S. eubayanus*
245 *SeMALT1* maltose symporter (*ScTEF1*$_p$-*SeMALT1-ScCYC1*$_t$) was PCR amplified from pUD794 using
246 primers 9036/9039, which included a 5' extension homologous to the co-transformed transporter
247 fragment and an extension homologous to the downstream region of the *ScSGA1* locus, respectively.
248 To facilitate integration in strain IMZ616, the two PCR fragments were co-transformed with plasmid
249 pUDR119 (*amdS*), which expressed a gRNA targeting *ScSGA1* (spacer sequence: 5'-
250 ATTGACCACTGGAATTCTTC-3') (van Rossum et al. 2016). The plasmid and repair fragments were
251 transformed using the LiAc yeast transformation protocol (Gietz and Schiestl 2007) and transformed
252 cells were plated on $SM_{Ace}G$. Correct integration was verified by diagnostic PCR with primers pairs
253 4226/4224. Strains *S. cerevisiae* IMX1704, IMX1706 and IMX1708 were constructed following the
254 same principle, but instead of using pUD794 to generate the transporter fragment, pUD795, pUD796
255 and pUD797 were used to PCR amplify *ScTEF1*$_p$-*SeMALT2-ScCYC1*$_t$, *ScTEF1*$_p$-*SeMALT3-ScCYC1*$_t$ and
256 *ScTEF1*$_p$-*SeAGT1-ScCYC1*$_t$ respectively. IMX1313 was constructed in a similar way using only *ScTEF1*$_p$-
257 *ScMAL31-ScCYC1*$_t$ amplified with primer pair 9036/11018 which contain 5'and 3' extensions
258 homologous to the upstream and downstream region of the *ScSGA1* locus. Correct integration was
259 verified by diagnostic PCR with primer pair 4226/4224 (Supplementary Figure 1). All PCR-amplified

260 gene sequences were Sanger sequenced (BaseClear). IMX1313 was grown on YPD to loose pUDR119
261 (*URA3*) and pUDC156 (*amdS*). An isolate unable to grown on SMG without uracil and with acetamide
262 was selected and named IMX1313Δ. This strain was able to grow on SMG supplemented with 150 mg
263 L^{-1} uracil.

264 To assess functionality of CDFM21L.1 *SeMALS1*, IMX1313Δ was transformed with 100 ng pUDE843
265 (*ScTDH3*$_p$-*SeMALS1-ScADH1*$_t$) by electroporation (Gorter de Vries et al. 2017), resulting in strain
266 IMZ752. Transformants were selected on SMG plates after 5 days of incubation at 20 °C and
267 validated by PCR (DreamTaq polymerase, Thermo Scientific) using primer pair 14454/14455
268 (Supplementary Figure 1). Similarly, functionality of the *SeMALS2* maltase gene of CDFM21L.1 was
269 assessed by transforming IMX1313Δ with pUDE844 (*ScTDH3*$_{pr}$-*SeMALS2-ScADH1*$_{ter}$), resulting in strain
270 IMZ753.

271 *S. eubayanus* IMK820 (*SemalT1Δ*) was constructed by transforming CDFM21L.1 with 200 ng of
272 pUDP091 and 1 µg of 120 bp repair fragment obtained by mixing an equimolar amount of primers
273 12442/12443, as previously described (Gorter de Vries et al. 2017). As a control, the same
274 transformation was performed without including the repair DNA fragment. Transformants were
275 selected on $SM_{Ace}G$ plates. *S. eubayanus* IMK823 (*Seagt1Δ*) was constructed similarly, using pUDP090
276 and primer pair 11320/11321. Deletion of *SemalT1* was verified by PCR with primer pair
277 11671/11672 and Sanger sequencing. The *Seagt1* deletion was verified by PCR using primer pair
278 12273/12274, and by Illumina whole-genome sequencing and read alignment to the reference
279 genome of CDFM21L.1 (Bioproject accession number PRJNA528469).

280 Strains IMX1765, IMX1939 and IMX1940 were constructed by inserting *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ at
281 the *SeSGA1* locus of CDFM21L.1, IMK820 and IMK823, respectively. A repair fragment containing
282 *ScPGK1*$_p$-*ScMAL13-ScTEF2*$_t$ was amplified from pUDE780 with primer pair 12917/12918. Strains
283 CDFML21L.1, IMK820 and IMK823 were transformed by electroporation by addition of 350 ng of
284 repair fragment and 560 ng pUDP052 (*amdS*) into the cells as previously described (Gorter de Vries et

285 al. 2017). Transformants were plated on $SM_{Ace}G$ and incubated at 20 °C. IMX1762 was constructed
286 similarly using a repair fragment with *ScTDH3*$_p$-*ScMAL12-ScADH1*$_t$ amplified from pUDE044 with
287 primer pair 12319/12320. Strains were verified by PCR using primer pair 12635/12636 and Sanger
288 sequencing.

289 **Hybrid Construction**

290 The *S. cerevisiae* X *S. eubayanus* hybrid HTSH020 was constructed by spore-to-spore mating . The *S.*
291 *eubayanus* strain CDFM21L.1 and the *S. cerevisiae* strain CBC-1 were grown in 20 mL YPD at 20 °C
292 until late exponential phase. Cells were centrifuged for 5 min at 1000 g and washed twice in
293 demineralized water. Cells were re-suspended in 20 mL sporulation medium and incubated for 64 h
294 at 20 °C. Presence of spores was verified by microscopy. Asci were harvested by centrifugation for 5
295 min at 1000 g, and washed with demineralized water, resuspended in 100 µL demineralized water
296 containing 100 U/mL of Zymolyase (MP Bio, Santa Ana, CA) and incubated for 10 min at 30 °C. Spores
297 were washed and plated on the edge of a YPD agar plate. Spores from the two strains were brought
298 in contact with each other with an MSM System 400 micromanipulator (Singer Instruments, Watchet,
299 United Kingdom). Zygote formation was observed after 6-8 h. Emerging colonies were re-streaked
300 twice on SM 2% maltose at 12 °C. Successful hybridization was verified by multiplex PCR using
301 DreamTaq DNA polymerase (Thermo Scientific), by amplifying the *S. cerevisiae* specific *MEX67* gene
302 with primer pairs 8570/8571 and by amplifying the *S. eubayanus* specific gene *SeFSY1* with primers
303 8572/8573 (Supplementary Figure 2), as previously described (Pengelly and Wheals 2013).

304 **Illumina sequencing**

305 Genomic DNA of *S. eubayanus* strains CDFM21L.1 and ABFM5L.1, *S. cerevisiae* strain CBC-1 and *S.*
306 *cerevisiae* x *S. eubayanus* strain HTSH020 was isolated as previously described (Van den Broek et al.
307 2015). Paired-end sequencing (2 X 150 bp) was performed on a 350 bp PCR-free insert library using
308 Illumina HiSeq2500 (San Diego, CA) by Novogene(HK) Company Ltd (Hong Kong, China). Genomic
309 DNA of the strains CBC-1 and HTSH020 was sequenced in house on a MiSeq sequencer (Illumina)

310 with 300 bp paired-end reads using PCR-free library preparation. Sequence data are available at NCBI
311 under Bioproject accession number PRJNA528469.

312 **MinION long read sequencing**

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

322 **De novo *assembly***

323 *De novo* assembly of the Oxford Nanopore MinION dataset was performed using Canu (v1.4, setting:
324 genomesize=12m) (Koren et al. 2017). Assembly correctness was assessed using Pilon (Walker et al.
325 2014) and further corrected by "polishing" of sequencing/assembly errors by aligning Illumina reads
326 with BWA (Li and Durbin 2010) using correction of only SNPs and short indels (–fix bases parameter).
327 For HTSH020, an artificial reference genome was made by combining the assembly of CBC-1 and
328 CDFM21L.1. The genome assemblies were annotated using the MAKER2 annotation pipeline (version
329 2.31.9) (Holt and Yandell 2011), using SNAP (version 2013–11-29) (Korf 2004) and Augustus (version
330 3.2.3) (Stanke et al. 2006) as *ab initio* gene predictors. *S. cerevisiae* S288C EST and protein sequences
331 were obtained from SGD (*Saccharomyces* Genome Database, http://www.yeastgenome.org/) and
332 were aligned using BLASTX on the obtained polished sequence assembly (BLAST version 2.2.28+)
333 (Camacho et al. 2009). Predicted translated protein sequences of the final gene model were aligned
334 to the *S. cerevisiae* S288C protein Swiss-Prot database using BLASTP (http://www.uniprot.org/).

335 Custom-made Perl scripts were used to map systematic names to the annotated gene names. Error
336 rates in nanopore-sequencing data were estimated from the q score (Phred scaled) per read, as
337 calculated by the base caller Albacore (version 1.1.0) (Oxford Nanopore Technology). Average q score
338 was used to calculate the error $P= 10^{q/10}$.
339
340 **RNA isolation**
341 CDFM21L.1, IMX1765, IMX1939 and HTS020 were grown in SMG, SMM and SMMt until mid-
342 exponential phase (OD_{660nm} of 12 for SMG/SMM and of OD_{660nm} 15 for SMMt). Culture samples
343 corresponding to ca. 200 mg of biomass dry weight were directly quenched in liquid nitrogen. The
344 samples were processed and total RNA extracted as previously described (Tai et al. 2005). Prior to
345 cDNA synthesis, purity, concentration and integrity of the RNA in the samples was assessed with
346 Nanodrop (Thermo Scientific), Qubit (Thermo Scientific) and Tapestation 220 with RNA Screen Tape
347 (Agilent Technologies), respectively, according the manufacturers' recommendations. cDNA libraries
348 were prepared using the TruSeq RNA V2 kit (Illumina). Paired-end sequencing (2 X 150 bp) was
349 performed on a 300 bp PCR-free insert library on a HISeq 2500 (Illumina) at Novogene (HK) Company
350 Ltd (Hong Kong, China). Duplicate biological samples were processed, generating an average
351 sequence quantity of 23.7M reads per sample. Reads were aligned to the CDFM21L.1 reference
352 assembly (GEO (https://www.ncbi.nlm.nih.gov/geo/) accession number GSE133146) using a two-pass
353 STAR (Dobin and Gingeras 2016) procedure. In the first pass, splice junctions were assembled and
354 used to inform the second round of alignments. Introns between 15 and 4000 bp were allowed, and
355 soft clipping was disabled to prevent low-quality reads from being spuriously aligned. Ambiguously-
356 mapped reads were removed from the dataset. Expression level for each transcript were quantified
357 using htseq-count (Anders et al. 2015) in union mode. Fragments per kilo-base of feature (gene) per
358 million reads mapped (FPKM) values were calculated by "Applying the fpkm method" from the edgeR
359 package (Robinson et al. 2010; McCarthy et al. 2012). Differential expression analysis was performed
360 using DESeq (Anders et al. 2013).

361

362 ## Results

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

365 It has been proposed that the *S. eubayanus* genetic pool of *S. pastorianus* was inherited from an
366 ancestor of the Asian *S. eubayanus* lineage (Bing et al. 2014). With 99.82% identity, the Himalayan *S.*
367 *eubayanus* strains CDFM21L.1 and ABFM5L.1 that belong to the Holarctic lineage (Peris et al. 2016),
368 present the closest characterized relatives of the *S. eubayanus* ancestor of lager brewing yeasts.
369 However, this distance was based on a limited sequencing space (Bing et al. 2014) and the analysis
370 did not investigate the presence of specific *S. eubayanus* genetic markers found in *S. pastorianus*
371 hybrids. Therefore, the genome of the Himalayan *S. eubayanus* strain CDFM21L.1 was sequenced
372 with a combination of long-read and short-read techniques (Oxford Nanopore MinION and Illumina
373 technologies, respectively) to generate a near-complete draft reference genome sequence. The
374 resulting CDFM21L.1 genome assembly comprised 19 contigs, including its mitochondrial genome. All
375 chromosomes were completely assembled from telomere to telomere, except for chromosome XII,
376 which was fragmented into 3 contigs due to the repetitive rDNA region and manually assembled into
377 a single scaffold. With a total size of 12,034,875 bp, this assembly represents the first near-complete
378 draft genome of a *S. eubayanus* strain of the Holarctic clade (Peris et al. 2016).

379 Chromosome-level assemblies were hitherto only available for the Patagonia B-clade strain CBS
380 12357^{T} (Libkind et al. 2011; Brickwedde et al. 2018). We identified three major structural differences
381 in CDFM21L.1 relative to CBS12357^{T} using Mauve (Darling et al. 2004): (i) a paracentric inversion in
382 the sub-telomeric region of chromosome VII, involving approximately 8 kbp, (ii) a translocation of
383 approximately 12 kbp from the left sub-telomeric region of chromosome VIII to the right sub-
384 telomeric region of chromosome VI, and (iii) a reciprocal translocation between approximately 20
385 kbp from the right sub-telomeric region of chromosome V and approximately 60 kbp from the center

386 of chromosome XII (Figure 1A). All structural variation involved sub-telomeric regions, in accordance
387 with their known relative instability (Brown et al. 2010; Moller et al. 2018; Gordon et al. 2009).
388 An alignment comparison of the CDFM21L.1 and CBS 12357^{T} genomes with MUMmer revealed that
389 557 kb were unique to CDFM21L.1 and reciprocally 428 kb were unique to CBS 12357^{T}. Sequences
390 unique to CBS 12357^{T} (3.6% of its genome) and to CDFM21L.1 (4.6% of its genome) were located
391 primarily in sub-telomeric regions and in repetitive regions, such as rDNA on chromosome XII (Figure
392 1B). Out of the 32 sub-telomeric regions, 23 exhibited absence of synteny. Conserved synteny was
393 observed for sub-telomeric regions on CHRIII (left), CHRIV (left and right), CHRVI (left), CHRIX (right),
394 CHRXI (right), CHRXII (right), CHRXIV (left) and CHRXV (right) (Supplementary File 1).
395 The 428 kb of sequence that were absent in the Himalyan *S. eubayanus* strain included 99 annotated
396 ORFs (Supplementary File 1). Of the 99 ORFs that were (partly) affected, 11 were completely absent
397 in CDFM21L.1, involving mostly genes implicated in iron transport facilitation (Supplementary File 1).
398 The 557 kb of sequence that was not present in CBS 12357^{T} included 113 annotated ORFs
399 (Supplementary File 1). Of these 113 ORFs, 15 were completely absent in CBS 12357^{T}. These 15 ORFs
400 showed an overrepresentation of genes involved in transmembrane transport (Fishers exact test, P-
401 value 4.8E^{-5}) (Figure 1C).
402 Of the 15 ORFs unique to CDFM21L.1, three were identical orthologs of *S. cerevisiae MAL11/AGT1*.
403 These three ORFs were found in the sub-telomeric regions of chromosomes VII, XIV and XV. Their
404 sequence similarity with the *S. cerevisiae* CEN.PK113.7D and *S. pastorianus* CBS 1483 *MAL11/AGT1*
405 genes was 82.7% and 99.89%, respectively. In addition to these *SeAGT1* genes, CDFM21L.1 genome
406 sequence harbored three maltose transporters (*SeMALTx*), two maltases (*SeMALSx*) and three
407 regulators (*SeMALRx*) encoding genes. In contrast to the situation in *S. eubayanus* CBS 12357^{T}, none
408 of the *SeMAL* genes formed a canonical *MAL* locus in CDFM21L.1 (Figure 2). A systematic sequence
409 inspection of these CDFM21L.1 *SeMAL* genes revealed mutations that prematurely interrupted the
410 reading frames of *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and *SeMALT*3 (CHRXII).

411 In addition to *S. eubayanus* CDFM21L.1 strain, a second Himalayan *S. eubayanus* isolate (ABFM5L.1)
412 was sequenced. These two strains were 99.97% genetically identical at the nucleotide level, their
413 *MAL* genes were syntenic and the premature stop codons in *SeMALR1* (CHRV), *SeMALT2* (CHRXII) and
414 *SeMALT*3 (CHRXII) were conserved. Two additional mutations were identified in one of the three
415 *SeAGT1* genes. A nucleotide variation on position 53 and 939 (T instead of an A and A instead of a G))
416 resulted in a glycine to valine and arginine to lysine change, respectively.

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

418 Identification of *SeAGT1* in the two Himalayan *S. eubayanus* strains suggests an ability to not only
419 grow on maltose but also on maltotriose. Strains from the Holarctic clade have previously been
420 hypothesized to be the donor of the *S. eubayanus* sub-genome in *S. pastorianus* hybrids (Bing et al.
421 2014; Peris et al. 2016). However, no physiological data regarding their ability to grow on the sugars
422 present in wort are available. To assess their growth characteristics, the *Asian S. eubayanus* strains
423 CDFM21L.1 and ABFM5L.1, the Patagonian *S. eubayanus* type strain CBS 12357^{T} and the *S.*
424 *pastorianus* strain CBS 1483 were grown on diluted industrial brewer's wort at 12 ˚C. As reported
425 previously, *S. pastorianus* strain CBS 1483 could utilize all three sugars but did not fully consume
426 maltotriose (Figure 3) (Brickwedde et al. 2017). Also in accordance with previous observations (Hebly
427 et al. 2015), CBS 12357^{T} consumed glucose and maltose completely, but left maltotriose untouched.
428 However, in marked contrast to *S. eubayanus* CBS 12357^{T}, neither CDFM21L.1 nor ABFM5L.1
429 consume maltose after growth on glucose. Moreover, like CBS 12357^{T}, maltotriose was not
430 metabolized by these two *S. eubayanus* strains. While in CBS 12357^{T} an ability to grow on maltose
431 and an inability to grow on maltotriose could be readily attributed to its *MAL* genes complement,
432 CDFM21L.1 and ABFM5L.1 failed to grow on maltose even though they appeared to contain
433 complete genes encoding maltose (*SeMALT1* and *SeAGT1*) and maltotriose (*SeAGT1*) transporters.
434

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

437 The recent characterization of maltose metabolism in CBS 12357^{T} showed that the coding regions of
438 transcriptionally silent maltose-transporter genes in *S. eubayanus* can potentially encode functional
439 proteins (Brickwedde et al. 2018). The inability of the *S. eubayanus* Himalayan isolates to grow on α-
440 oligosaccharides precluded direct testing of transporter-gene functionality by deletion studies.
441 Instead, these genes were expressed in *S. cerevisiae* IMZ616, which is devoid of all native maltose
442 metabolism genes (Marques et al. 2018). The CDFM21L.1 transporter genes *SeMALT1, SeMALT2,*
443 *SeMALT3* or *SeAGT1* were integrated at the *ScSGA1* locus in IMZ616 along with the *S. cerevisiae*
444 maltase gene *ScMAL12* (Brickwedde et al. 2018), yielding a series of strains overexpressing a single
445 transporter (IMX1702 (*SeMALT1*), IMX1704 (*SeMALT2*), IMX1706 (*SeMALT3*) and IMX1708 (*SeAGT1*)).
446 These strains, as well as the negative and positive control strains IMZ616 and IMX1365 (IMZ616
447 expressing *ScAGT1* and *ScMAL12*), were grown on SM media supplemented with either maltose or
448 maltotriose. On maltose, not only the positive control strain IMX1365, but also IMX1702 (*SeMALT1*)
449 and IMX1708 (*SeAGT1*) were able to grow on maltose, consuming 30 and 60%, respectively, of the
450 initially present maltose after 100 h (Figure 4A). As anticipated, the *SeMALT2* and *SeMALT3* alleles
451 with premature stop codons did not support growth on maltose. Of the two strains that grew on
452 maltose, only IMX1708 (*SeAGT1*) also grew on maltotriose. These results demonstrate that *SeAGT1*
453 from a Holarctic *S. eubayanus* encoded a functional maltotriose transporter and, consequently, that
454 the inability of Holarctic strains to grow on maltose and maltotriose was not caused by transporter
455 dysfunctionality.

456 In addition to transport, metabolism of α-oligoglucosides requires maltase activity. Functionality of
457 the putative *SeMALS1* and *SeMALS2* maltase genes was tested by constitutive expression in strain
458 IMZ616, together with a functional *Sc*MAL31 transporter genes, yielding strains IMZ752 and IMZ753,
459 respectively. The maltase-negative strain IMX1313 was used as negative control. In SM medium with

460 maltose, both IMZ752 (*SeMALS1*) and IMZ753 (*SeMALS2*) grew and completely consumed maltose
461 within 65 h, demonstrating functionality of both hydrolase genes (Figure 4C).
462 In *S. cerevisiae* transcriptional regulation of *MALx2* and *MALx1* genes is tightly controlled by a
463 transcription factor encoded by *MALx3* genes. Malx3 binds an activating site located in the
464 bidirectional promoters that control expression of *MALx2* and *MALx1* genes (Meurer et al. 2017;
465 Levine et al. 1992). To test whether absence of maltose consumption in Himalayan *S. eubayanus*
466 strains was caused by a lack transcriptional upregulation of *SeMALT* and *SeMALS*, the *S. cerevisiae*
467 *ScMAL13* gene was integrated at the *SeSGA1* locus in *S. eubayanus* CDFM21L.1, under the control of
468 a constitutive *ScPGK1* promoter and *ScTEF2* terminator. *ScMAL13* expression in CDFM21L.1 enabled
469 growth on maltose and maltotriose (Figure 5A), indicating that a lack of transcriptional upregulation
470 was indeed the cause of the parental strain's inability to grow on these oligoglucosides. However,
471 consumption of maltose and maltotriose was incomplete and consumed sugars were almost
472 exclusively respired, as no ethanol was measured after 60 h of cultivation.

473 The possibility to grow an engineered variant of *S. eubayanus* CDFM21L.1 on α-oligoglucosides
474 offered an opportunity to study transporter function in its native context. Complementary functional
475 characterization by gene deletion of *SeMALT1* and *SeAGT1* was performed using CRISPR-Cas9
476 genome editing method (Brickwedde et al. 2018; Gorter de Vries et al. 2017). Deletion of *SeMALT1*
477 and *SeAGT1* in CDFM21L.1 resulted in strains IMK820 and IMK823, respectively. Complete deletion of
478 *SeAGT1* required disruption of six alleles. To confirm the complete removal of all copies, the genome
479 of IMK823 was sequenced. Mapping reads onto the reference *S. eubayanus* CDFM21L.1 genome
480 assembly confirmed that all six alleles were removed simultaneously. Subsequently, the regulator
481 expression cassette ($ScPGK1_p$-*ScMAL13*-$ScTEF2_t$) was integrated in IMK820 and IMK823 at the *SeSGA1*
482 locus yielding strains IMX1939 and IMX1940, respectively (Figure 5B). The four deletion strains
483 IMK820 (*SemalT1*Δ), IMK823 (*Seagt1*Δ), IMX1939 (*SemalT1*Δ *Sesga1*Δ::*ScMAL13*) and IMX1940
484 (*Seagt1*Δ *Sesga1*Δ::*ScMAL13*) were characterized on SMG, SMM or SMMt. All four strains were able
485 to grow on glucose (Supplementary Figure 3). While strains IMK820, IMK823 and IMX1940 were

486 unable to grow on maltose or maltotriose (Figure 5C), strain IMX1939 (*SemalT1*Δ
487 *Sesga1*Δ::*SeMAL13*), which harbored functional *SeAGT1* copies, grew on maltose as well as on
488 maltotriose. However, after 64 h of growth, these sugars were only partially consumed. Only 1.2 g L^{-1}
489 ethanol was produced from maltose and no ethanol formation was observed during growth on
490 maltotriose. The low ethanol concentration and the relatively high OD_{660nm} suggest that, under the
491 experimental conditions strain IMX1939 exhibited a Crabtree negative phenotype and exclusively
492 respired maltotriose. *S. eubayanus* IMX1940 (*Seagt1*Δ *Sesga1*Δ::*SeMAL13*) did not consume
493 maltotriose after 84 h of incubation. Moreover, despite the presence of *SeMALT1*, which encoded a
494 functional maltose transporter upon expression in *S. cerevisiae* IMZ616, strain IMX1940 was also
495 unable to consume maltose.

496 In addition to a functional Malx3 transcription factor, transcriptional activation of *MAL* genes also
497 requires presence of a cis-regulatory motif in the promoter of regulated genes. Transcriptome
498 analysis of *S. eubayanus* CBS 12357^T recently showed that absence of a canonical cis-regulatory motif
499 in *SeMALT1* and *SeMALT3* of *S. eubayanus* CBS 12357^T caused a deficiency in their expression
500 (Brickwedde et al. 2018). To further explore regulation of *SeMAL* and *SeAGT1* genes, we investigated
501 the impact of carbon sources on genome-wide transcriptome and, specifically, on transcriptional
502 activation of genes involved in maltose metabolism. Duplicate cultures of *S. eubayanus* strain
503 IMX1765 ($ScPGK1_p$-*ScMAL13*-$ScTEF2_t$) were grown on SMG, SMM and SMMt at 20 °C and sampled in
504 mid-exponential phase. After mRNA isolation and processing, cDNA libraries and reads were
505 assembled onto the newly annotated *S. eubayanus* CDFM21L.1 genome to calculate FPKM
506 (fragments per kilobase of feature (gene) per million reads mapped) expression values. The
507 heterologous regulator *ScMAL13*, expressed from the constitutive *ScPGK1* promoter, displayed the
508 same expression in glucose- and maltose-grown cultures. Although *ScMAL13* was efficiently
509 expressed on glucose, none of the nine *S. eubayanus* maltose genes (the three identical *SeAGT1*
510 copies being undistinguishable) were transcriptionally induced under these conditions (Figure 6),
511 confirming that the hierarchical regulatory role of glucose catabolite repression (Vidgren et al. 2011;

512 Meurer et al. 2017) also takes place in *S. eubayanus*. During growth on maltose, all nine genes were
513 significantly upregulated relative to glucose-grown cultures but large variations in expression level
514 were observed. The maltase genes *SeMALS1* and *SeMALS2* and the transporter gene *SeAGT1* showed
515 the highest upregulation, with fold changes of 148, 161 and 2355 respectively. Although upregulated
516 *SeMALT1* displayed a fold change of 13, its normalized expression in maltose-grown cultures was
517 886-fold lower than that of *SeAGT1*. This weaker upregulation might explain why, despite the ability
518 of its coding region to support synthesis of a functional maltose transporter, *SeMALT1* alone could
519 not restore growth on maltose. The transcriptome data also revealed that the absence of maltose
520 induction in CDFM21L.1 was not associated with defective cis-regulatory elements in *SeMALR*
521 promoter sequences since the regulator genes were properly activated, instead these results would
522 suggest that the *Se*MalR regulator are not functional.

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

525 The genetic make-up of *S. pastorianus* lager-brewing yeasts strongly advocates that they originate
526 from hybridization of *S. cerevisiae* and *S. eubayanus* parental lineages that were both unable to
527 metabolize maltotriose (Nakao et al. 2009). This hypothesis is consistent with the recurrent mutation
528 in the *S. cerevisiae AGT1* allele of *S. pastorianus* strains as well as with the inability of Himalayan
529 strains of *S. eubayanus* to grow on these oligoglucosides.

530 Spores of the Himalayan *S. eubayanus* CDFM21L.1 were hybridized with *S. cerevisiae* CBC-1. This top-
531 fermenting *S. cerevisiae* is recommended for cask and bottle conditioning and unable to consume
532 maltotriose (Lallemand, Montreal, Canada). Analysis of the CBC-1 assembly, obtained by a
533 combination of long and short read sequencing, linked its maltotriose-negative phenotype to a total
534 absence of the *MAL11/AGT1* gene. The resulting laboratory interspecific hybrid HTSH020 was
535 characterized at 12 °C on synthetic wort, a defined medium whose composition resembles that of
536 brewer's wort. While *S. eubayanus* CDFM21L.1 only consumed glucose and *S. cerevisiae* CBC-1
537 consumed glucose and maltose after 103 h (Figure 7A), the interspecific hybrid HTSH020 completely

538 consumed glucose, maltose and partially consumed maltotriose after 105 h, thus resembling
539 characteristics of *S. pastorianus* strains (e.g. CBS1483 (Brickwedde et al. 2017)). In addition to this
540 gain of function, the hybrid HTSH020 outperformed both its parents on maltose consumption, since
541 it depleted this sugar in 70 h instead of 95 h for strain CBC-1. Since *S. cerevisiae* grows generally
542 slower at 12 °C, the experiments were also performed at 20 °C where HTSH020 consumed all maltose
543 16 h earlier than CBC-1 (Supplementary Figure 4).
544 Transcriptome analysis of the hybrid strain HTSH020 grown on SM with different carbon sources
545 showed that *SeAGT1* expression was repressed during growth on glucose, with a normalized
546 expression level of 7 FPKM (Figure 7B). When grown on SM maltose, *SeAGT1, SeMALS1* and *SeMALS2*
547 were significantly induced, with fold increases of 816, 109 and 116, respectively (Figure 7B). Although
548 *SeMALT1* and *SeMALT2* were induced, these transporters do not contribute to maltose metabolism
549 due to truncation of their ORFs. These transcriptome data demonstrated that *SeAGT1* and *SeMALS*
550 genes are induced by regulatory crosstalk between regulators encoded from the CBC-1 *S. cerevisiae*
551 sub-genome and maltotriose transporter genes harbored by the *S. eubayanus* genome. This
552 laboratory hybridization experiment may be the closest reproduction yet of how, centuries ago,
553 maltotriose-fermentation capacity arose in the first hybrid ancestor of *S. pastorianus*.
554

555 ## Discussion

556 The ability to consume maltose and maltotriose represents a key performance indicator of *S.*
557 *pastorianus* lager-brewing strains (Vidgren et al. 2009). This study demonstrates how mating of *S.*
558 *cerevisiae* and *S. eubayanus* strains that cannot themselves ferment maltotriose, can yield
559 maltotriose-fermenting hybrids. This laboratory study illustrates how, centuries ago, maltotriose-
560 fermentation capacity may have arisen in the first hybrid ancestor of *S. pastorianus*.
561 While the origin of the *S. eubayanus* parent of *S. pastorianus* strains is still under debate
562 (Dunn and Sherlock 2008; Okuno et al. 2016; Monerawela and Bond 2017), phylogenetic analysis

563 suggested a Far East Asian origin (Bing et al. 2014). However, this interpretation was based on a
564 limited sequencing space and was constrained by the quality of available sequence assemblies. Since
565 an ortholog of *SeAGT1* had previously only been found in the *S. eubayanus* sub-genome of *S.*
566 *pastorianus* strains, this finding revived the discussion on the geographical origin of the ancestral *S.*
567 *eubayanus* parent (Baker and Hittinger 2018). The high-quality, annotated genome assemblies of the
568 Himalayan *S. eubayanus* strains CDFM21L.1 and ABFM5L.1 presented in the present study revealed
569 several copies of *SeAGT1*, whose very high sequence identity with *S. pastorianus SeAGT1* are
570 consistent with the previously proposed Asian origin of the S. *eubayanus* sub-genome of *S.*
571 *pastorianus* (Bing et al. 2014; Baker and Hittinger 2018; Peris et al. 2016). Next, genome-sequence
572 comparison of the Patagonian B sub-clade *S. eubayanus* strain CBS12357^{T} and the Holarctic sub-clade
573 strain CDFM21L.1 revealed homoplasy of *SeAGT1*, probably reflecting that these sub-clades evolved
574 in different ecological niches.

575 The *S. eubayanus* wild stock whose genome sequence most closely corresponds to the *S.*
576 *eubayanus* sub-genome of *S. pastorianus* originates from the Tibetan plateau of the Himalaya (Bing
577 et al. 2014). However, the first *S. cerevisiae x S. eubayanus* hybrid, from which current lager yeasts
578 evolved by centuries of domestication, likely originates from a region between Bavaria and Bohemia
579 in Central Europe. So far, European *S. eubayanus* isolates have not been reported. This may indicate
580 that the original hybridization event occurred elsewhere or that the ancestral European lineage went
581 extinct. The recent detection, in a metagenomics analysis of samples from the Italian Alps, of ITS1
582 sequences corresponding to *S. eubayanus* could indicate that a wild European lineage exists after all
583 (Alsammar et al. 2019).

584 Functional characterization by heterologous complementation of an *S. cerevisiae* mutant
585 strain established that the *Se*Agt1 transporters from the Himalayan *S. eubayanus* strains CDFM21L.1
586 and ABFM5L supported uptake of maltose and maltotriose. After showing that these strains also
587 encoded a functional maltase gene, their inability to grow on maltose and maltotriose was attributed
588 to an inability to transcriptionally upregulate maltose metabolism genes likely caused by loss of

589 function mutations the regulator. In *S. cerevisiae* and, to some extent, ,in *S. eubayanus* strains of the
590 Patagonian B sub-clade such as CBS12357^{T} (Baker et al. 2015; Brickwedde et al. 2018; Vidgren et al.
591 2005), MAL loci exhibit a specific organization in which a transporter (*MALT*) and a hydrolase (*MALS*)
592 gene are expressed from the same bidirectional promoter and are located adjacent to a regulator
593 gene (*MALR*) (Charron et al. 1989). In contrast, of the seven genomic regions harboring *MAL* genes in
594 the two Asian *S. eubayanus* strains, none showed this canonical organization (Figure 2) and the
595 subtelomeric regions carrying *SeAGT1* did not harbour sequences similar to hydrolase or regulator
596 genes. Subtelomeric regions harboring the other *MAL* genes indicated intensive reorganization as a
597 result of recombination. In particular, subtelomeric regions on CHRII, CHRV and CHRXII provide clear
598 indications for recombination events that scattered genes from ancestral MAL1 and MAL2 loci over
599 several chromosomes. A similar interpretation could explain the reorganization MAL3 on CHRXIII
600 (Figure 2). Similar events may have contributed to loss of function of the MAL regulators (MalR), as
601 exemplified by the occurrence of a non-synonymous mutation in S*eMALR1* resulting in loss of
602 function. These rearrangements did, however, not inactivate the *cis*-regulatory sequences of the
603 *MAL* genes, since complementation with a functional *ScMAL13* allele caused induction of most
604 *SeMAL* genes (Figure 6, Figure 7B) and, thereby, the heterotic maltotriose-positive phenotype of the
605 hybrid strain HTSH020. Together with the high copy number of *SeAGT1*, this heterotic
606 complementation may have been the main driver for colonization of low-temperature brewing
607 processes by the early hybrid ancestors of current *S. pastorianus* strains.
608 Recent work on adaptation to brewing environments of laboratory *S. cerevisiae* × *S. eubayanus*
609 hybrids showed loss maltotriose utilization during serial transfer in wort (Gorter de Vries et al. 2018).
610 A similar loss of maltotriose utilization is frequently encountered in *S. cerevisiae* ale strains (Gallone
611 et al. 2016), as well as in some Saaz-type *S. pastorianus* strains (Gibson et al. 2013). This is thus in
612 contrast with retention of a maltotriose assimilation phenotype by Frohberg-type *S. pastorianus*
613 strains. This may have been facilitated by the occurrence of multiple copies of the *SeAGT1* gene in
614 the *S. eubayanus* ancestor, which could act as a sequence buffer to counteracting adverse effects of

615 gene copy loss. The recent release of the first long-read sequencing assembly of *S. pastorianus*
616 enabled a precise chromosomal mapping of the maltose-metabolism genes (Salazar et al. 2019) and
617 showed that the Frohberg type *S. pastorianus* strains CBS 1483 harbored one copy of *SeAGT1* on the
618 *S. eubayanus* CHRXV section (as in CDFM21L.1) of the chimeric chromosome formed from *Se*CHRXV
619 and *Se*CHRVIII (Salazar et al. 2019).
620 Differential retention and loss of maltotriose consumption in *S. pastorianus* lineages may reflect
621 different brewing process conditions during domestication. In modern brewing processes based on
622 high-gravity wort, cell division is largely constrained to the glucose and maltose phases, which occur
623 before depletion of nitrogen sources (Lei et al. 2012). It may be envisaged that, in early lager-brewing
624 processes, unstandardized mashing processes generated wort with a higher maltotriose content,
625 which would have allowed for continued yeast growth during the maltotriose consumption phase.
626 During serial transfer on sugar mixtures, the selective advantage of consuming a specific sugar from a
627 mixture correlates with the number of generations on that sugar during each cycle (Papapetridis et
628 al. 2018; Wisselink et al. 2009). Such conditions would therefore have conferred a significant
629 selective advantage to a maltotriose-assimilating *S. cerevisiae* x *S. eubayanus* hybrid, especially if,
630 similar to current ale yeasts, the *S. cerevisiae* parent was unable to ferment maltotriose.
631 The heterotic phenotype that was reconstructed in the interspecies *S. cerevisiae* x *S.*
632 *eubayanus* hybrid HTS020 resulted from combination of dominant and recessive genetic variations
633 from both parental genomes. *S. eubayanus* contributed the *SeAGT1* gene and its functional *cis*-
634 regulatory sequences, but also harbored recessive mutations in *MALR* genes that allowed full
635 expression of the heterotic phenotype. These mutations were complemented with a set of *S.*
636 *cerevisiae* genes including a functional *MALR* and a non-functional *ScAGT1* gene to match the
637 mutations found in *S. pastorianus* (Nakao et al. 2009). Although some *S. pastorianus* strains harbor
638 an additional maltotriose transporter encoded by *SpMTT1 (*Dietvorst *et al. 2005)*, this gene was
639 recently proposed to have emerged after the original hybridization event as a result of repeated
640 recombination between *MALT* genes from both sub-genomes (Brouwers et al. 2018).

641 Maltotriose fermentation is likely not the only heterotic phenotype of S. *pastorianus* strains.
642 Flocculation or formation of complex aroma profiles (Bolat et al. 2013; Vidgren and Gibson 2018) are
643 phenotypes that are not fully understood and difficult to reproduce, that also might result from
644 heterosis (Gorter de Vries et al. 2018).
645 Laboratory-made *S. cerevisiae* x *S. eubayanus* hybrids hold great potential for brewing process
646 intensification and for increasing product diversity. In addition to increasing our understanding of the
647 evolutionary history of lager yeast genomes evolutionary, this study has implications for the design
648 of new hybrids. Hitherto, laboratory crosses of *S. cerevisiae* x *S. eubayanus* strains were designed
649 based on combination of dominant traits of the parental strains. Our results show that recessive
650 traits can be just as important as contributors to the genetic diversity of such hybrids.
651

652 ## Accession Numbers

653 The sequencing data were deposited at NCBI (https://www.ncbi.nlm.nih.gov/) under the Bioproject
654 PRJNA528469 and the transcriptomics data were deposited at GEO (Genome Expression Omnibus
655 https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE133146.
656

657 ## Supplementary Data

658 Supplementary Data are available
659

660 ## Authors contributions

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

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Conflict of Interest

The authors declare no conflict of interest.

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919 **Table 1: *Saccharomyces* strains used in this study.** The abbreviation *malΔ* indicates *mal11-mal12*::loxP *mal21-mal22*::loxP
920 *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$^-$	(Bing et al. 2014)
ABFM5L.1	*S. eubayanus*	Wildtype Mal$^-$ Mtt$^-$	(Bing et al. 2014)
CBS 12357	*S. eubayanus*	Wildtype Mal$^+$ Mtt$^-$	(Libkind et al. 2011) 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*	(Entian and Kötter 2007)
IMZ616	*S. cerevisiae*	*MATa ura3-52 LEU2 MAL2-8C malΔ mp2/3hΔ suc2Δ ima1Δ* ima2Δ *ima3Δ ima4Δ ima5Δ* pUDC156 (*Spcas9 URA3 ARS4 CEN6*)	(Marques et al. 2018)
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*$_t$	(Brickwedde et al. 2018)
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

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

922

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

Plasmid	Relevant genotype	Origin
p426-TEF-amdS	*ori* (*ColE1*) *bla* 2µ amdSYM *TEF1*$_{pr}$-*CYC1*$_{ter}$	(Marques et al. 2017)
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*	(de Kok et al. 2011)
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*$_{ter}$	(Juergens et al. 2018)
pUDP004	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$-BsaI-BsaI-Sc*CYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Spcas9*$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	(Gorter de Vries et al. 2017)
pUDP052	ori (ColE1) *bla* panARSopt amdSYM *ScTDH3*$_{pr}$- gRNA$_{SGA1}$-*ScCYC1*$_{ter}$ *AaTEF1*$_{pr}$-*Sp*cas9$^{D147Y\ P411T}$-*ScPHO5*$_{ter}$	(Brickwedde et al. 2018)
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*$_{ter}$	(van Rossum et al. 2016)
pYTK074	ori (ColE1) *cat URA3*	(Lee et al. 2015)
pYTK082	*cat* 2µ	(Lee et al. 2015)
pYTK083	ori (ColE1) *bla*	(Lee et al. 2015)
pUD631	ori (ColE1) *bla* gRNA$_{SeMALT1}$	(Brickwedde et al. 2018)
pUD634	ori (ColE1) *bla* gRNA$_{SeAGT1}$	This study
pUDC156	ori (ColE1) *bla ARS4 CEN6 URA3 Spcas9*	(Marques et al. 2018)

924

925

926 **Legends to Figures**

927 **Figure 1. Genome comparison between CDFM21L.1 and CBS 12357^T. (A)** Translocations in
928 CDFM21L.1 relative to CBS12357^T, (i) a paracentric inversion in the sub-telomeric region of
929 chromosome VII, involving approximately 8 kbp, (ii) a translocation of approximately 12 kbp from the
930 left sub-telomeric region of chromosome VIII to the right sub-telomeric region of chromosome VI,
931 and (iii) a reciprocal translocation between approximately 20 kbp from the right sub-telomeric region
932 of chromosome V and approximately 60 kbp from the center of chromosome XII.Red lines depicted
933 chromosomes separation. Genome syntheny is indicated with colored blocks. **(B)** Relative
934 chromosome position of gene presence differences between CDFM21L.1 (blue) and CBS 12357^T (red).
935 **(C)** Representation of the assembled CDFM21L.1 *S. eubayanus* chromosomes, the black boxes denote
936 newly added sequences. New annotated open reading frames and gene entries modified relative to
937 the CBS 12357T draft genome (Brickwedde et al. 2018).

938

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

952

953 **Figure 3. Characterization of sugar consumption of *S. pastorianus* CBS1483 (black) and *S.***
954 ***eubayanus* CBS 12357^T (blue), CDFM21L.1 (red), and ABFM5L.1 (green) on wort.** For every sample,
955 glucose (■), maltose (▲), maltotriose (▼) and ethanol (◆) were measured from the supernatant.
956 Strains were grown at 12 °C for 110 hours in infusion Neubor flasks. Samples were filtered through a
957 0.22 µm filter and analyzed on HPLC. Data represents average and standard deviation of three
958 biological replicates.

959

960 **Figure 4. Overexpression of *SeMALT*, *SeAGT1* and *SeMALS* genes in a maltose negative background**
961 ***S. cerevisiae* strain.** Maltose negative background strain IMZ616 (■), IMX1365 overexpressing
962 *ScMAL11* (▲), IMX1702 overexpressing *SeMALT1* (▼), IMX1704 overexpressing *SeMALT2* (◆)
963 IMX1706 overexpressing *SeMALT3* (●) and IMX1708 overexpressing SeAGT1 (□) were grown on SM
964 2% maltose or maltotriose at 20 °C. Growth on maltose **(A)** and on maltotriose **(B)** was monitored
965 based on optical density (OD_{660nm}) and concentrations of maltose and maltotriose in culture
966 supernatants were measured by HPLC. Data are presented as average and standard deviation of two
967 biological replicates. **(C)** IMX1313 overexpressing only *ScMAL31* (○), IMZ752 overexpressing
968 *ScMAL31* and *SeMALS1* (△) and IMZ753 overexpressing *ScMAL31* and *SeMALS2* (▽) grown on SM
969 maltose 2%. Growth was monitored based on optical density measurement at 660 nm (OD_{660nm}) and
970 maltose in culture supernatants was measured by HPLC. Data represents average and standard
971 deviation of two biological replicates.

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

977 constructed knockout strains. Knockouts of *SeMALT1* (IMK820) and *SeAGT1* (IMK823) were made
978 with CRISPR-Cas9. Subsequently the *SeSGA1* locus was replaced by $ScPGK1_p$- *ScMAL13-*$ScTEF2_t$ using
979 CRISPR-Cas9 in both strains resulting in IMX1939 and IMX1940, respectively. **(C)** *S. eubayanus* strains
980 IMK820 (■), IMK823 (▲), IMX1939 (▼) and IMX1940 (◆) were characterized on SM with maltose or
981 maltotriose at 20 °C. OD_{660nm} was measured (black) and sugar (black) and ethanol (red)
982 concentrations were determined from the supernatant by HPLC. All data represents average and
983 standard deviation of biological duplicates.

984

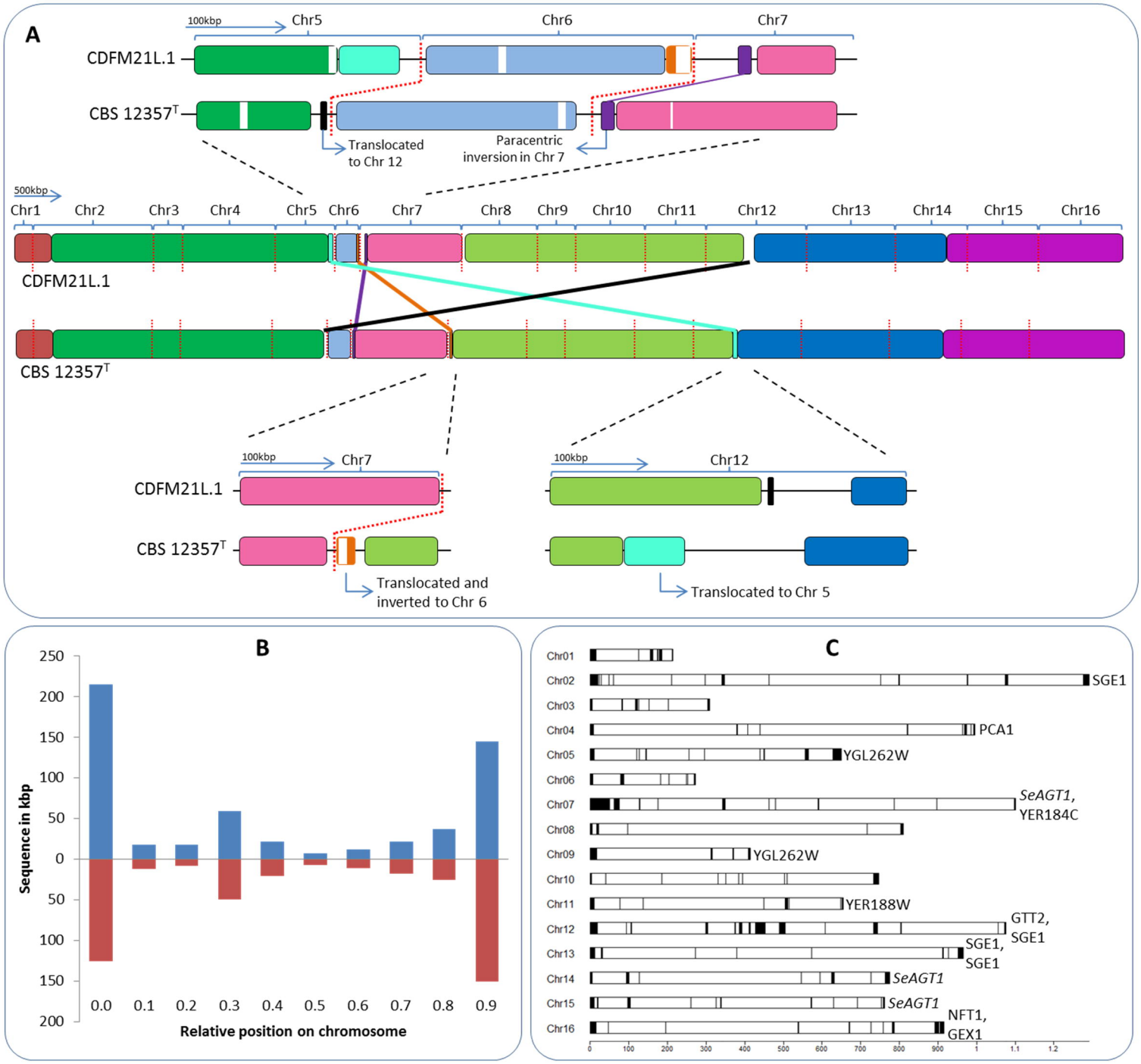
985 **Figure 6. Expression levels of maltose metabolism genes in IMX1765.** Normalized transcript levels of
986 maltose metabolism genes from IMX1765mid-exponential phase grown on glucose (red), maltose
987 (blue)and maltotriose (green) at 20 °C were calculated from duplicate RNA sequencing experiments
988 (2x 150 bp) using the FPKM method . All data represents average and standard deviation of two
989 biological duplicates.

990

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

1000

1001


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
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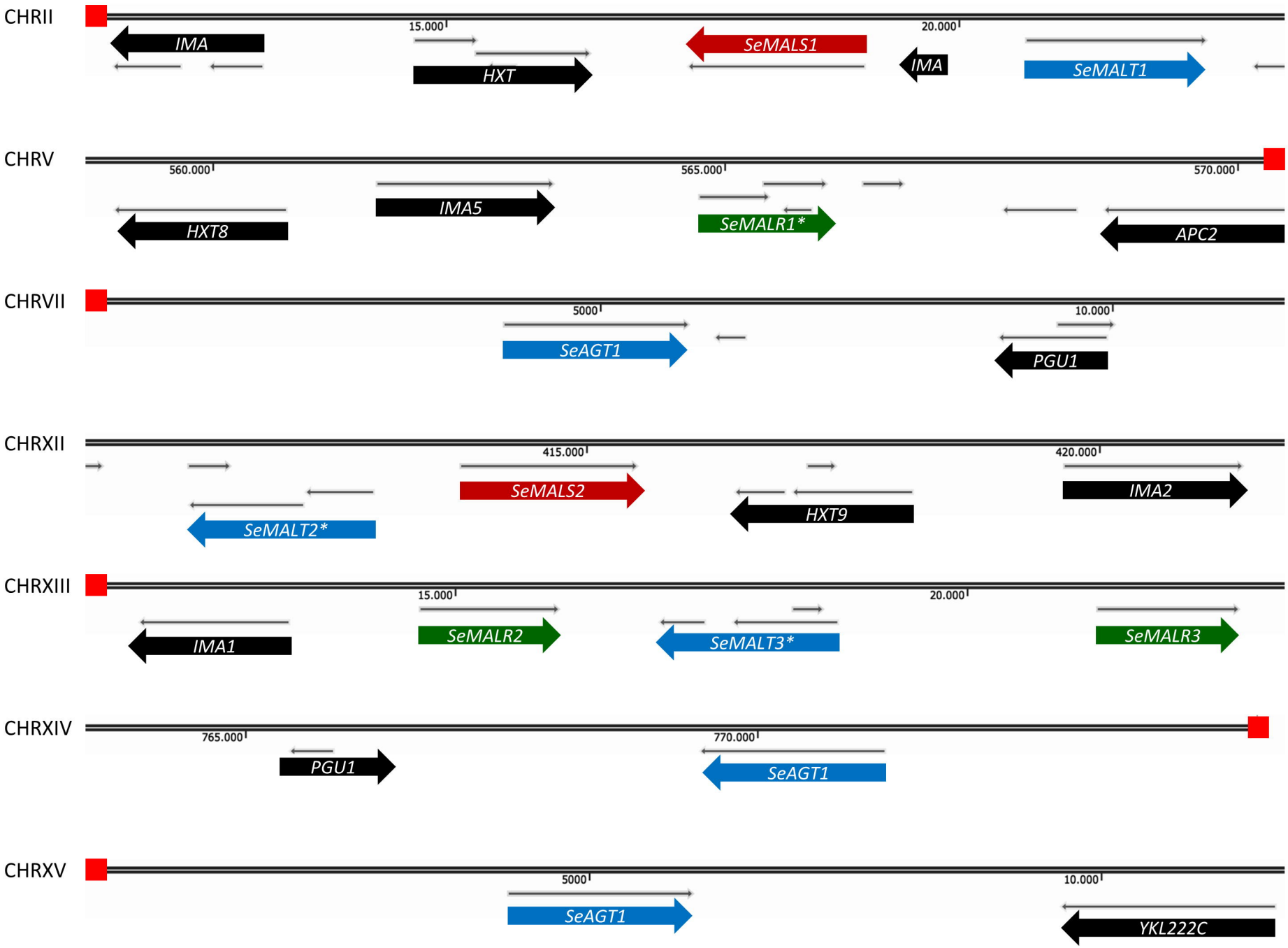
100
200
300
400
500
600
700
800
900
1
1.1
1.2


CHRII
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20.000
IMA
HXT
SeMALS1
IMA
SeMALT1
CHRV
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565.000
570.000
HXT8
IMA5
SeMALR1*
APC2
CHRVII
5000
10.000
SeAGT1
PGU1
CHRXII
415.000
420.000
SeMALS2
SeMALT2*
HXT9
IMA2
CHRXIII
15.000
20.000
IMA1
SeMALR2
SeMALT3*
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CHRXIV
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770.000
PGU1
SeAGT1
CHRXV
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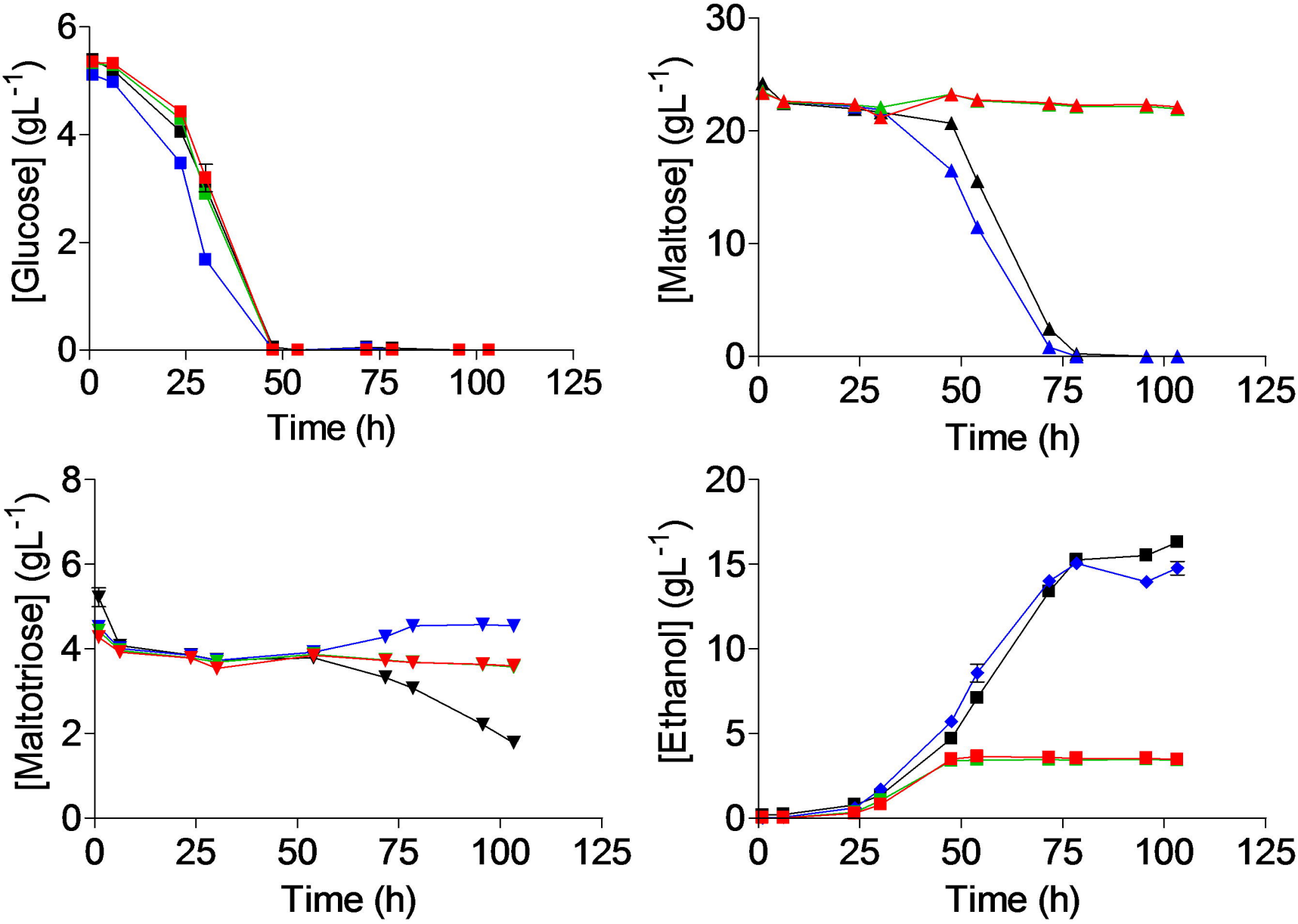
SeAGT1
YKL222C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Time (h)
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Time (h)
[Maltotriose] (gL-1)
Time (h)
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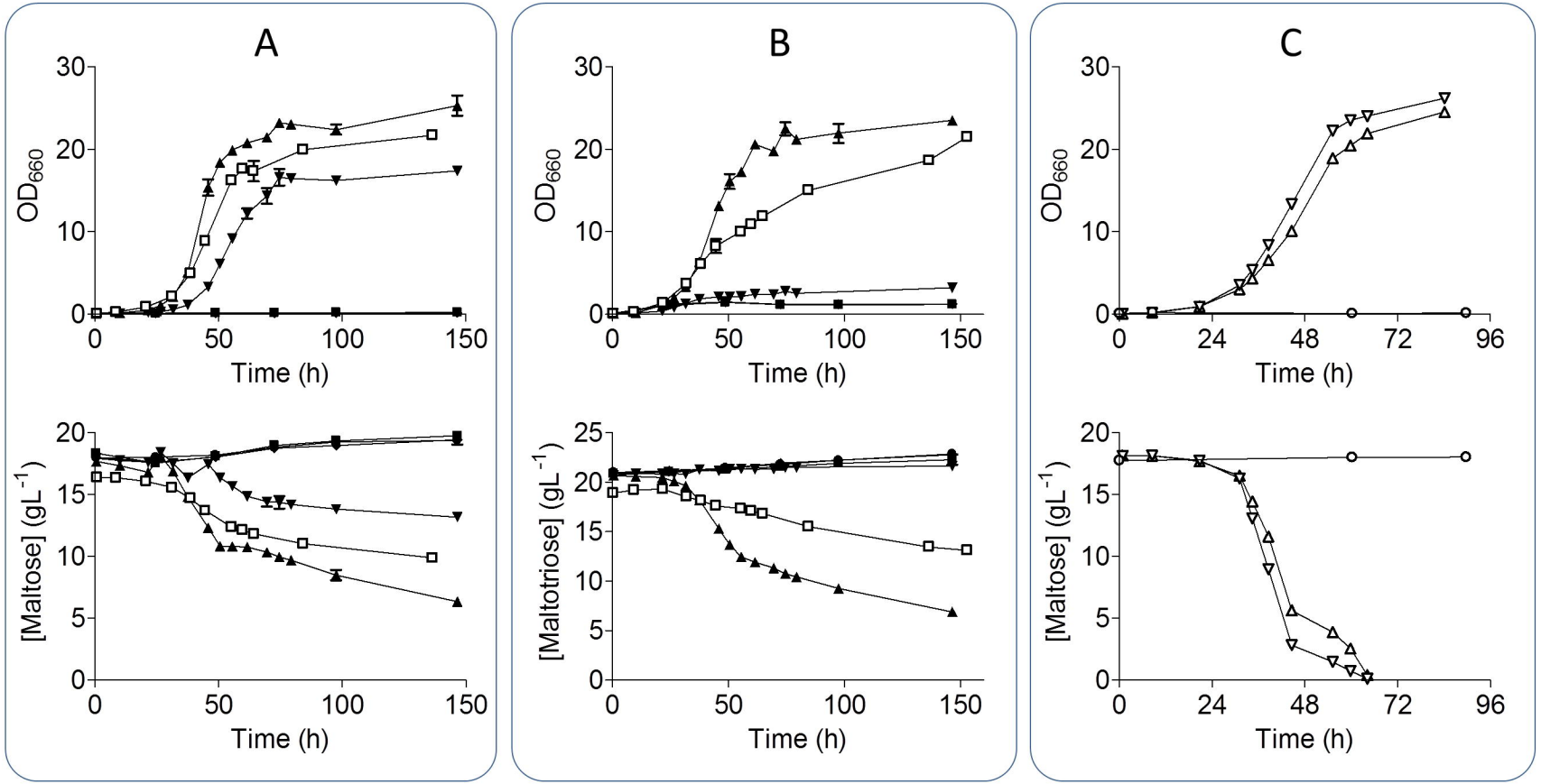
Time (h)


A
B
C
OD660
Time (h)
[Maltose] (gL-1)
[Maltotriose] (gL-1)
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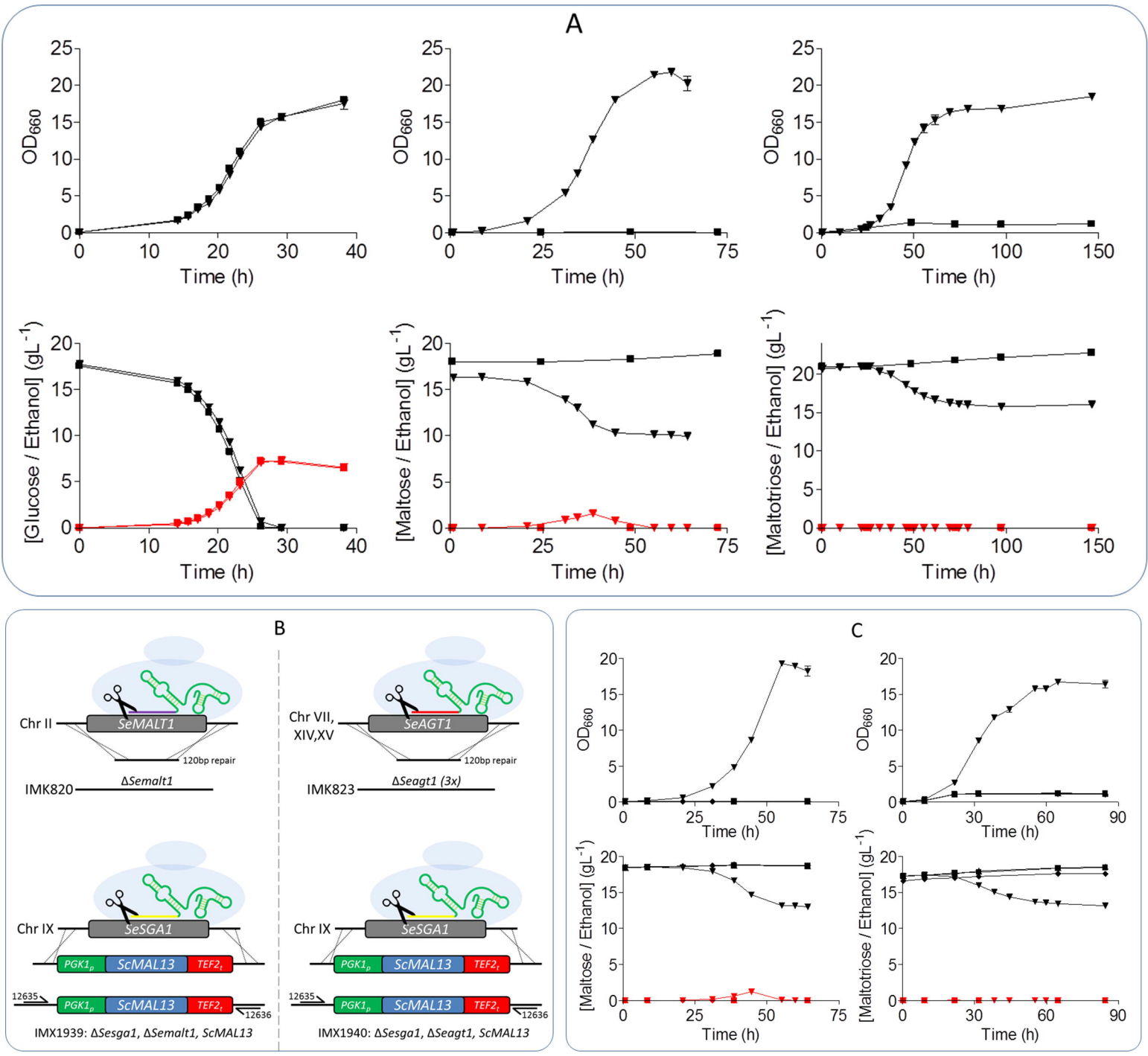
10
20
30
50
100
150
24
48
72
96
5
15
25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OD660
Time (h)
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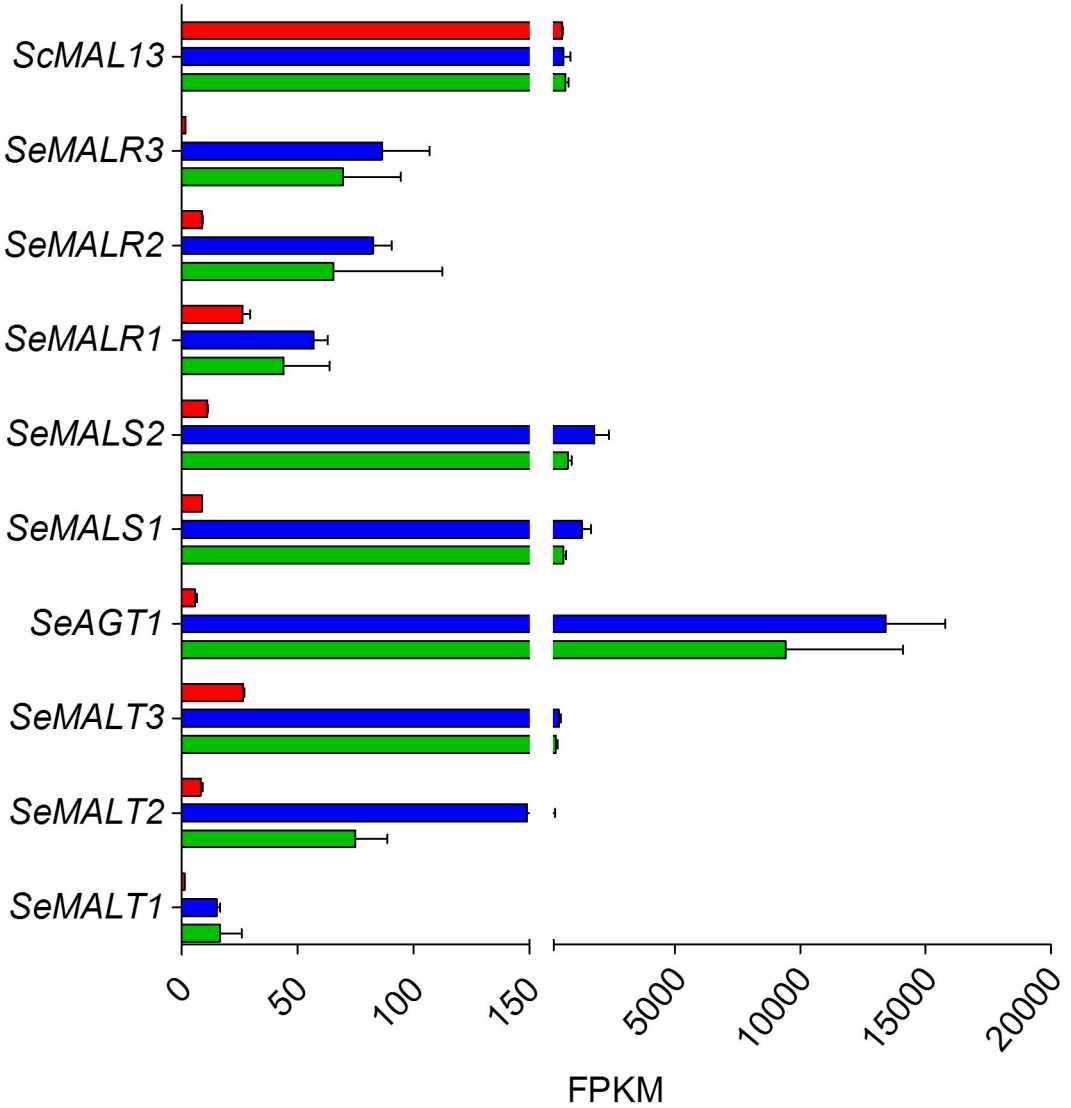
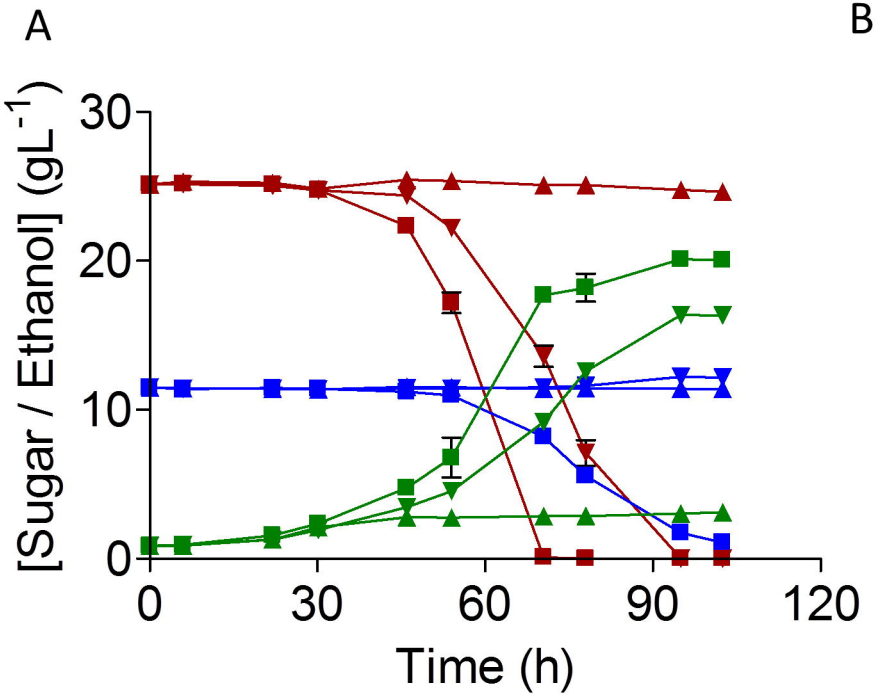
[Maltose / Ethanol] (gL-1)
[Maltotriose / Ethanol] (gL-1)
B
Chr II
SeMALT1
120bp repair
IMK820
ΔSemalt1
Chr VII, XIV,XV
SeAGT1
IMK823
ΔSeagt1 (3x)
Chr IX
SeSGA1
PGK1p
ScMAL13
TEF2t
12635
12636
IMX1939: ΔSesga1, ΔSemalt1, ScMAL13
IMX1940: ΔSesga1, ΔSeagt1, ScMAL13
C


ScMAL13
SeMALR3
SeMALR2
SeMALR1
SeMALS2
SeMALS1
SeAGT1
SeMALT3
SeMALT2
SeMALT1
0
50
100
150
5000
10000
15000
20000
FPKM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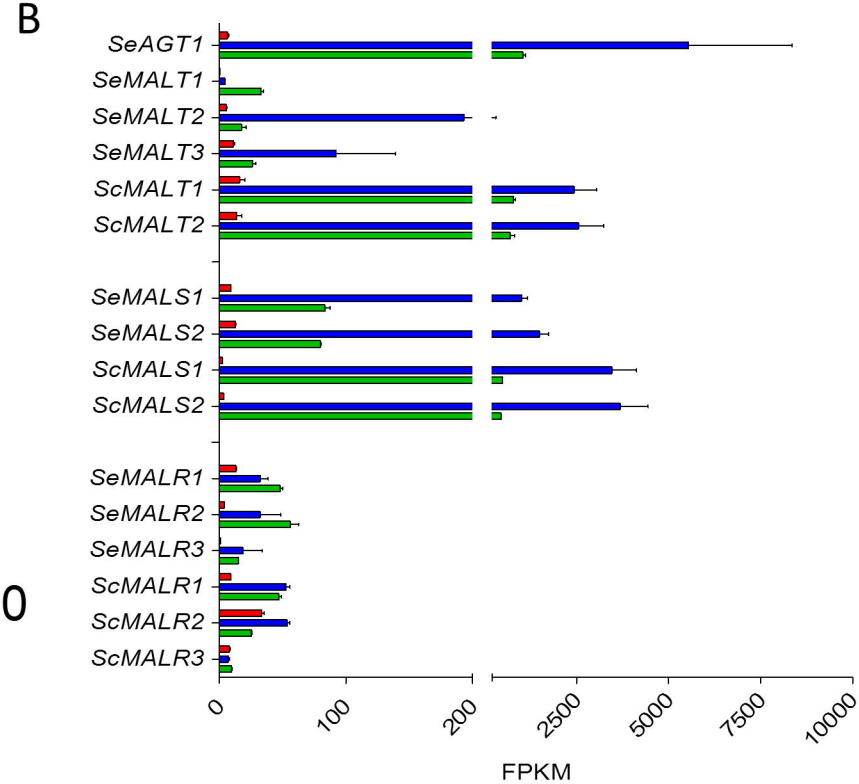
[Sugar / Ethanol] (gL-1)
30
20
10
0
0
30
60
90
120
Time (h)


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