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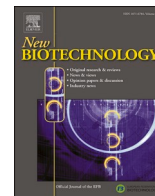
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Improving CRISPR-Cas9 mediated genome integration in interspecific hybrid yeasts

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ABSTRACT

Saccharomyces pastorianus is not a classical taxon, it is an interspecific hybrid resulting from the cross of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus*. Exhibiting heterosis for phenotypic traits such as wort α -oligosaccharide consumption and fermentation at low temperature, it has been domesticated to become the main workhorse of the brewing industry. Although CRISPR-Cas9 has been shown to be functional in *S. pastorianus*, repair of CRISPR-induced double strand breaks is unpredictable and preferentially uses the homoeologous chromosome as template, preventing targeted introduction of the desired repair construct. Here, we demonstrate that lager hybrids can be edited with near 100% efficiency at carefully selected landing sites on the chimeric *SeScCHRIII*. The landing sites were systematically selected and evaluated for (i) absence of loss of heterozygosity upon CRISPR-editing, (ii) efficiency of the gRNA, and (iii) absence of effect on strain physiology. Successful examples of highly efficient single and double gene integration illustrated that genome editing can be applied in interspecies hybrids, paving the way to a new impulse to lager yeast strain development.

Introduction

With an annual production of almost 2 billion hectolitres, lager beer is the world's most consumed alcoholic beverage (<https://www.statista.com/>). The workhorse for lager beer production is the yeast *Saccharomyces pastorianus*, a hybrid yeast that has emerged from a natural hybridization event, probably occurring during the Middle Ages, between the mesophilic ale yeast *Saccharomyces cerevisiae* and the cryotolerant yeast *Saccharomyces eubayanus* [1–3]. Hitherto most research aiming at improving the brewing performance of lager strains has been based on classical strain improvement [4–11]. Brewing strains resulting from these techniques are regarded as non-genetically modified organisms. Although these techniques proved their efficiency, they are time consuming and unpredictable, thus limiting the pace of strain

development. Conversely molecular techniques and targeted genetic modifications would offer a faster alternative to creating specific mutants that might exhibit better brewing characteristics. The deployment of genetic engineering improvement strategies in the brewing industry is, however, impeded by three factors. The first is the producers' concerns about consumer acceptance of beers brewed with genetically modified yeasts [12]. While it is difficult to predict whether these reservations will last, in North America, yeasts-producing companies are already marketing modified ale and even lager strains (<https://berkeleyyeast.com/>, <https://www.lallemandbrewing.com/>) demonstrating that opinions are changing. The second point concerns the limited genetic accessibility of these hybrid yeasts, a trait likely related to the complex structure of their genome. Thirdly, the breweries have to deal with the regulatory implications for production with genetically

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; CHR, CHROmosome; gRNA, guide-RNA; DSB, Double Strand Break; LOH, Loss Of Heterozygosity; YPD, Yeast extract Peptone Dextrose; LB, Lysogeny Broth; HH, HammerHead; HDV, Hepatitis Delta Virus; FSC, Forward Scatter; SSC, Side Scatter; FACS, Fluorescence-Activated Cell Sorting; HPLC, High-Performance Liquid Chromatography; GC, Gas Chromatography; FID, Flame Ionization Detector; WGS, Whole Genome Sequencing.

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modified organisms (GMOs), depending on their location in the world.

The hybrid nature of the *S. pastorianus* genome was confirmed for the first time with the genome sequence of the *S. pastorianus* Weihenstephan 34/70 strain [1]. This initial work was followed by multiple other *S. pastorianus* strain sequencing projects [13–16] that confirmed that lager yeast harbored an allo-aneuploidy genome. Although strain to strain variations exist, the lager yeast genome comprises both parental chromosome sets which all have different copy numbers. In addition to the presence of multiple copies, each chromosome has a homoeologous chromosome, which derives from the other parental species [17]. For example, the *S. cerevisiae* CHRI is a homoeolog of the *S. eubayanus* CHRI, which shares both high sequence similarity and gene synteny [16,18]. This co-occurrence has likely contributed to translocation and loss of heterozygosity (LOH) events between chromosomes of the two sub-genomes (ScSe and SeSc) in *S. pastorianus* that are considered as hallmarks of domestication [15,19].

While this dual genome composition has promoted the emergence of essential phenotypic traits of lager brewing yeasts, the presence of homoeologous chromosome pairs has impaired genetic amenability [20]. This effect is even stronger when genome editing tools such as CRISPR-Cas9 are used. CRISPR-Cas endonucleases can simply target specific DNA sequences based on the user-defined composition of the single guide RNA (gRNA) sequence and presence of the protospacer adjacent motif (PAM) [21–24]. The CRISPR-Cas induced double strand breaks (DSB) are extremely recombinogenic and are resolved by the yeast homology directed repair (HDR) machinery, that uses native or foreign DNA containing sequence homology as repair template, thereby producing the desired genetic edit [25].

Although the CRISPR-Cas9 methodology has been successfully applied in *S. pastorianus* [26], it appeared in many situations that the editing outcome is unpredictable and characterization of the resulting edited locus has proven troublesome [20]. Whereas, in homozygous diploid yeasts, the gRNA programmed Cas9 introduces DSBs on both copies, in hybrid yeasts, gRNA are not necessarily designed to cut at all parental alleles. In this latter situation, the uncut homoeologous locus competes with the provided repair DNA fragments, facilitating chromosome recombination and reduced editing efficiency, resulting in loss of heterozygosity and even more undesired genetic changes [20].

The aim of this study is to improve the genetic accessibility of the allo-aneuploid yeast *S. pastorianus* by identifying unique landing sites devoid of homoeologous regions using a systematic approach. We evaluate whether the approach eliminates LOH when performing precise genome editing with CRISPR-Cas9. Identifying and targeting unique sequences offers opportunities for increased genetic engineering efficiencies in allo-aneuploid yeast. To demonstrate this, integration efficiency of single and double genes is tested.

Table 1
Strains used in this study.

Species	Strain	Relevant genotype	Origin
<i>S. cerevisiae</i>	CEN.PK113–7D	MATa <i>MAL2–8c SUC2</i>	[69]
<i>S. pastorianus</i>	CBS 1483	Group II brewer's yeast, Heineken bottomyeast, isolated July 1927	[70]
<i>S. pastorianus</i>	WS 34/70-AE1	Group II brewer's yeast, flocculent yeast	[1]
<i>S. pastorianus</i>	IMK1062	CBS 1483 Δ ScEEB1::ymNeongreen (LOH), colony 1	This study
<i>S. pastorianus</i>	IMK1063	CBS 1483 Δ ScEEB1::ymNeongreen (LOH), colony 3	This study
<i>S. pastorianus</i>	IMK1064	CBS 1483 Δ ScEEB1::ymNeongreen (LOH), colony 4	This study
<i>S. pastorianus</i>	IMK1065	CBS 1483 Δ ScEEB1::ymNeongreen (LOH), colony 6	This study
<i>S. pastorianus</i>	IMI504	CBS 1483 Δ SeYCL049C::ymNeongreen	This study
<i>S. pastorianus</i>	IMI505	CBS 1483 Δ SeYCL036W::ymNeongreen	This study
<i>S. pastorianus</i>	IMI506	CBS 1483 Δ SeYCL012C::ymNeongreen	This study
<i>S. pastorianus</i>	IMI507	CBS 1483 Δ ScYCR051W::ymNeongreen	This study
<i>S. pastorianus</i>	IMI508	CBS 1483 Δ ScYCR087C-A::ymNeongreen	This study
<i>S. pastorianus</i>	IMI509	CBS 1483 Δ SeSite1::ymNeongreen	This study
<i>S. pastorianus</i>	IMI510	CBS 1483 Δ SeSite2::ymNeongreen	This study
<i>S. pastorianus</i>	IMI511	CBS 1483 Δ SeSite3::ymNeongreen	This study
<i>S. pastorianus</i>	IMI512	CBS 1483 Δ SeSite4::ymNeongreen	This study
<i>S. pastorianus</i>	IMI483	CBS 1483 Δ ScYCR087C-A::TDH3p-BbaldC-ENO1t	This study
<i>S. pastorianus</i>	IMI485	CBS 1483 Δ ScYCR087C-A::TDH3p-LlaldC-ENO1t	This study

Materials and methods

Strains and cultivation conditions

All yeast strains used in this study are listed in Table 1. Yeasts were grown on complex medium that contained 10 g·L⁻¹ bacto yeast extract, 20 g·L⁻¹ bacto peptone and 20 g·L⁻¹ glucose (YPD). For selection of transformants or when selective pressure was required, YPD medium was supplemented with 200 mg·L⁻¹ of hygromycin (YPD hyg). Solid YPD media was obtained by addition of 20 g·L⁻¹ bacto agar. *S. cerevisiae* and *S. pastorianus* strains were cultivated at 30 °C and 20 °C, respectively while shaking at 200 rpm in an Innova 43/43 R incubation shaker (Brunswick, Nijmegen, the Netherlands). On solid media, *S. cerevisiae* and *S. pastorianus* strains were cultivated on agar plates at 30 °C and 20 °C, respectively. Fermentations were performed with full malt wort at either 17° P or 5.7° P (Heineken, Zoeterwoude, The Netherlands) supplemented with 1 mL·L⁻¹ pluronic acid.

Escherichia coli XL1-Blue (New England Biolabs, Ipswich, MA, USA) cultures were grown in 15 mL Greiner tubes containing 5 mL lysogenic broth (LB) medium supplemented with 10 mg·L⁻¹ ampicillin (LB Amp), 50 mg·L⁻¹ kanamycin (LB Kan) or 10 mg·L⁻¹ chloramphenicol (LB Cam) for selection and cultivated at 37 °C. Solid medium was prepared by addition of 20 g·L⁻¹ bacto agar to the medium. Frozen stock cultures of *S. cerevisiae*, *S. pastorianus* and *E. coli* strains were prepared by addition of 30% (v/v) glycerol and stocks were stored as 1 mL aliquots at – 80 °C.

Molecular biology techniques

PCR amplifications for cloning purposes and diagnostic PCRs were performed as described in [20]. All plasmids and primers (Sigma Aldrich, Zwijndrecht, The Netherlands) constructed or used in this study are listed in Tables 2 and 3, respectively. Golden Gate cloning was performed using 25 fmol of each fragment with BsmBI or BsaI restriction enzymes (New England Biolabs) according to [27,28]. Gibson Assembly was performed with 200 fmol of each fragment using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) and one hour incubation at 50 °C [29]. Constructed plasmids were transformed into *E. coli* XL1-blue (New England Biolabs) chemically competent cells.

CRISPR-Cas9 plasmid construction

The gRNA sequences for the introduction in iCas9 platform plasmid pUDP002 [30] were designed in such a way that they yield compatible sticky ends for ligation upon restriction with BsaI, following the Golden Gate cloning strategy for gRNA cloning described in [26]. The plasmids pUD1205-pUD1209, pUD1243, pUD1244, pUD1212 and pUD1213

Table 2
Plasmids used in this study.

Plasmid	Genotype ^a	Reference
pUDP002	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p _{Bsal-Bsal} -ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	[30]
pUD1205	ori kanR ^{Bsal} HH-gRNA _{SeYCL049C} -HDV ^{Bsal}	GeneArt
pUD1206	ori kanR ^{Bsal} HH-gRNA _{SeYCL036W} -HDV ^{Bsal}	GeneArt
pUD1207	ori kanR ^{Bsal} HH-gRNA _{SeYCL012C} -HDV ^{Bsal}	GeneArt
pUD1208	ori kanR ^{Bsal} HH-gRNA _{ScYCR051W} -HDV ^{Bsal}	GeneArt
pUD1209	ori kanR ^{Bsal} HH-gRNA _{ScYCR087C-A} -HDV ^{Bsal}	GeneArt
pUD1243	ori kanR ^{Bsal} HH-gRNA _{SeSite1} -HDV ^{Bsal}	GeneArt
pUD1244	ori kanR ^{Bsal} HH-gRNA _{SeSite2} -HDV ^{Bsal}	GeneArt
pUD1212	ori kanR ^{Bsal} HH-gRNA _{SeSite3} -HDV ^{Bsal}	GeneArt
pUD1213	ori kanR ^{Bsal} HH-gRNA _{SeSite4} -HDV ^{Bsal}	GeneArt
pUDP168	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{ScEEB1} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP172	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{ScEAT1} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP269	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeYCL049C} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP270	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeYCL036W} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP271	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeYCL012C} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP272	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{ScYCR051W} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP273	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{ScYCR087C-A} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP283	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeSite1} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP284	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeSite2} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP276	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeSite3} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pUDP277	ori ampR panARS ^(OPT) AgTEF1p-hph-AgTEF1t ScTDH3p-HH-gRNA _{SeSite4} -HDV-ScCYC1t AaTEF1p-Spycas9 ^{D147Y P411T} -ScPHO5t	This study
pYTK009	ori camR TDH3p	[27]
pYTK051	ori camR ENO1t	[27]
pYTK053	ori camR ADH1t	[27]
pGGKp304	ori camR ymNeogreen	[31]
pGGKd015	ori ampR ConLS-GFPdo-ConR1	[71]
pGGKd034	ori ampR 2 μm hygR ConLS-GFPdo-ConR1	This study
pUDE1111	ori ampR 2 μm hygR ConLS-TDH3p-ymNeogreen-ADH1t-ConR1	This study
pUDE1112	ori ampR 2 μm hygR ConLS-TDH3p-ymScarletI-ADH1t-ConR1	This study
pUD1218	ori ampR TDH3p-BaldC-ENO1t	This study
pUD1220	ori ampR TDH3p-LaldC-ENO1t	This study

(GeneArt, Thermo Fisher Scientific, Waltham, MA) contain the gRNA sequences targeting SeYCL049C, SeYCL036W, SeYCL012C, ScYCR051W, ScYCR087C-A, SeSite1, SeSite2, SeSite3 and SeSite4, respectively, and are flanked by the HammerHead (HH) and Hepatitis Delta Virus (HDV) ribozymes. Correct Golden Gate cloning of these gRNAs into the platform plasmid pUDP002 resulted in pUDP269-pUDP273, pUDP283, pUDP284, pUDP276 and pUDP277, which were verified by diagnostic PCR with primer 4068 binding to SHR I and primers 18516, 18521, 18526, 18531, 18536, 18541, 18546, 18551, 18556, 19067 and 19068 binding to the respective gRNA spacers. Cloning of the gRNAs targeting ScEEB1 and ScEAT1 into pUDP002, resulting in pUDP168 and pUDP172 was confirmed in a diagnostic PCR with primers 1153 and 580.

The plasmids pUDE1111 and pUDE1112 that express the ymNeogreen gene and the ymScarletI gene respectively, were constructed using

Golden Gate cloning with plasmids pYTK009, pYTK053 [27], pGGKp034 (ymNeogreen [31]) or pGGKp032 (ymScarletI [31]) respectively, and pGGKd034 (Table 2). Correct construction of pUDE1111 and pUDE1112 was verified by diagnostic PCR with primers 10320 & 10325.

Construction of acetolactate decarboxylase gene expressing plasmids

aldC genes from *Brevibacillus brevis* (CP087980.1), excluding the secretion signal peptide, and *Leuconostoc lactis* (CP042420.1) were codon optimized for yeast, and ordered as synthetic genes (GeneArt, Thermo Fisher Scientific), resulting in plasmids pUD374 and pUD376. Genes were amplified from these plasmids using primer pairs 18759 & 18760 and 18763 & 18764, respectively, incorporating golden gate compatible flanks. Golden gate assembly with pGGKd015, pYTK009, pYTK051 and the PCR-amplified *aldC* gene fragment resulted in plasmids pUD1218 and pUD1220, respectively, for *BaldC* and *EaldC*, which were verified by diagnostic PCR using the primer pair 10320 & 10325.

CRISPR-Cas9 genome editing in *S. pastorianus* and *S. cerevisiae* strains

CRISPR-Cas9 genome editing in *S. pastorianus* strains was performed by transforming CBS 1483 or WS 34/70 with 500 ng gRNA plasmid and 1000 ng repair fragment (Table 4). As negative control, transformation from which the repair fragment was omitted was performed. Yeast transformation was performed by electroporation as described in [20]. The transformed cells were incubated in 0.5 mL YPD during 2 h, followed by re-suspending in 100 μL sterile demi-water and plating on selective medium. CRISPR-Cas9 genome editing in *S. cerevisiae* strain CEN.PK113-7D was performed by transforming 500 ng gRNA plasmid and 1000 ng repair fragment according to the LiAc/ss-DNA/PEG chemical transformation method [32].

The ymNeogreen integrations were performed by co-transforming the gRNA plasmid and the corresponding repair fragment consisting of the ymNeogreen expression cassette flanked by 60 bp homologous sequences for recombination. The ymNeogreen repair fragments were obtained by PCR amplification using pUDE1111 as template and primer pairs 18512 & 18513 for SeYCL049C, 18517 & 18518 for SeYCL036W, 18522 & 18523 for SeYCL012C, 18527 & 18528 for ScYCR051W, 18532 & 18533 for ScYCR087C-A, 18537 & 18538 for SeSite1, 18542 & 18543 for SeSite2, 18547 & 18548 for SeSite3 and 18552 & 18553 for SeSite4. The *aldC* gene expression cassettes for integration in YCR087C-A were obtained by PCR amplification with primers 18532 & 18533 using pUD1218 or pUD1220, respectively, as template.

In vivo assembled integration of ymNeogreen and ymScarletI was performed with two fragments to repair the CRISPR-induced DSB. The ymNeogreen fragment was amplified from pUDE1111 with primers 18512 & 18768 incorporating the SeYCL049C flank and the SHR CB flank, while the ymScarletI fragment was amplified from pUDE1112 with primers 13047 & 18513 incorporating the SHR CB and SeYCL049C flanks.

Yeast genomic DNA for diagnostic purposes was isolated using the lithium acetate (LiOAc)-SDS method [33]. Gene integrations in SeYCL049C were confirmed with primers 18514 & 18515, SeYCL036W with 18519 & 18520, SeYCL012C with 18990 & 18991, ScYCR051W with 18529 & 18530, ScYCR087C-A with 18534 & 18535, SeSite1 with 18539 & 18540, SeSite2 with 18544 & 18545, SeSite3 with 18549 & 18550, SeSite4 with 18992 & 18993, ScEEB1 with 15944 & 15945 and ScEAT1 with 15950 & 15951.

Flow cytometry and cell sorting

After the transformation procedure, the cell suspension was plated onto selective medium (1/5th of the cells) or transferred to 20 mL liquid selective medium in vented tubes (4/5th of the cells) and cultivated for 5

Table 3
Primers used in this study.

Number	Primer name	Sequence (5' -> 3')	Purpose
gRNA plasmid construction			
4068	Nic1_amp_Fwd	GCCTACGGTTCCCGAAGTATGC	Diagnostic primer gRNA cloning into pUDP002
18516	dg_YCL049C_gRNA	TGTCTCTGACTGTATCTGGGA	Diagnostic primer gRNA YCL049C
18521	dg_YCL036W_gRNA	CGGACTCCTCAATGATCAAA	Diagnostic primer gRNA YCL036W
18526	dg_YCL012C_gRNA	CGTCAATATAACTACATTTTGGGA	Diagnostic primer gRNA YCL012C
18531	dg_YCR051W_gRNA	ATACCCCGTTGCACCATG	Diagnostic primer gRNA YCR051W
18536	dg_YCR087C-A_gRNA	CACGCCATCTTCAAACGTCT	Diagnostic primer gRNA YCR087C-A
19067	dg_site1v2_gRNA	CGTATAGCACTCCTGCCGAA	Diagnostic primer gRNA Site 1
19068	dg_site2v2_gRNA	ACTACCCCTAGCACTGCTCA	Diagnostic primer gRNA Site 2
18551	dg_site3_gRNA	GTCGAGAAGATTTCTCGAAGATA	Diagnostic primer gRNA Site 3
18556	dg_site4_gRNA	CGTCATAATGAACAATCTCCAGTG	Diagnostic primer gRNA Site 4
1153	GPDp FW	GACCCACGCATGTATCTATCTC	Diagnostic primer gRNA ScEEB1 & ScEAT1
580	RPXKS1	GAATGTAAGCGTGACATAAC	Diagnostic primer gRNA ScEEB1 & ScEAT1
Construction expression cassettes (ymNeongreen, ymScarlet1, BbALC, LIALDC and EaALDC)			
10320	ConLS_Fw	CATGCCGGATGACACGAAC	Diagnostic primer YTK
10325	ConR1_Rv	AGTCATCCGAGCGTGTATTG	Diagnostic primer YTK
18759	Bb_aldB-CO-part3_Fw	AAAGCATCGTCTCATCGGTCTCATATGACTACTGTACTGTCCAGC	Amplification BbALDC
18760	Bb_aldB-CO-part3-Rv	AAAATGCCGTCTCAGGTCTCAGGATAGTTACTTTCTTCAGATTTCAGCTTGGTG	
18761	Ea_ald_CO-part3_Fw	AAAGCATCGTCTCATCGGTCTCATATGAACCACGCTTCTGACTGTAC	Amplification LIALDC
18762	Ea_ald_CO-part3-Rv	AAAATGCCGTCTCAGGTCTCAGGATAGTTAAGATTCAACAGATCTGATAGCAG	
18763	Ll_ald_CO-part3-Fw	AAAGCATCGTCTCATCGGTCTCATATGCTAGATTGTACCAACACGGTAC	Amplification EaALDC
18764	Ll_ald_CO-part3-Rv	AAAATGCCGTCTCAGGTCTCAGGATAGTTATTGTTGACCACCTTCAGAAGC	
Construction integration fragments			
18512	ConLS_YCL049C	TTCTATAGATGTACGTAAAGTTCTGCTTCTTTTATAGATAAAGAGATTGAGACTCGCCACTGGCCGATAATTGCAGACG	Integration in SeYCL049C
18513	ConR1_YCL049C	AGCTCAAGAAAAACCAGGCTATTTCGCAGAACAGGATAACCAGCTCTGTAAACCACATCAATCATGATGAGCCGTGATGACCC	
18517	ConLS_YCL036W	AGATGCGGCAGCCAGTGTAGGGCGTTGGGCATGATCGAAAGCCAAAGACCCACCAATTTCGAGACTGGCCGATAATTGCAGACG	Integration in SeYCL036W
18518	ConR1_YCL036W	ATATACATATACGTACCTATGTATCTACAGAAAGAAAAAAGATCAATGAATATATCATGATGAGCCGTGATGACCC	
18522	ConLS_YCL012C	CAAATTTGAAAGAACTAAAAAGACAGGAGGAAACCTCCCTTTTGTATGAGCTCAAAAATAAACCCTGGCCGATAATTGCAGACG	Integration in SeYCL012C
18523	ConR1_YCL012C	CCTCCCTATTCAAGGAAGAGAAATGAAAAATCTTCTTGCAGAAAGGTTGCAAACTACTATACATGATGAGCCGTGATGACCC	
18527	ConLS_YCR051W	GAGAACAAGAAGAGTTTGCAGGTGACAAAAATCGATGATTAAGTGTGTTGACGACAAAAACTGGCCGATAATTGCAGACG	Integration in ScYCR051W
18528	ConR1_YCR051W	TTCCATGTAGCGTCCGCTTCAATTTCTTTTGAAGCTATAAGAGCCCTTTGTGCTGGTCCGATGATGAGCCGTGATGACCC	
18532	ConLS_YCR087C-A	AAAAGATGAAACCAGTAAAGCTGCTACATAATGTCTATATCTACACATAAAAATCCGAACCTGGCCGATAATTGCAGACG	Integration in ScYCR087C-A
18533	ConR1_YCR087C-A	TAAGAGTATTCTGTATACAACAGCAACCGTCTCAGTCAAGAAATATTTGTTATTACAGCCATGATGAGCCGTGATGACCC	
18537	ConLS_Site1	CCTCCGCTATCTAGATGGATACAGAAAGCCGTTTTTTGGTGATTAATGTTCACTGTCAAGTACTGGCCGATAATTGCAGACG	Integration in SeSite1
18538	ConR1_Site1	CTTAAATTTGGAAGACTCCATCCAAGAGAAGGATCAGGTATCTGAATTTATCTAAAAGCCATGATGAGCCGTGATGACCC	
18542	ConLS_Site2	ACTCGATGACTCACAGGACAGGAGCGCTTTAAACAAGACCGGAATACATAAGCGCATGTGCACTGGCCGATAATTGCAGACG	Integration in SeSite2
18543	ConR1_Site2	AAATTAATCTTCTGTATATAAAAAGACTAGTACGCTTCTATACGTATATTTATTTCTTACATGATGAGCCGTGATGACCC	
18547	ConLS_Site3	AAAGCACACCGCTGATTTGGTTATATGACACCCAAATGGTAAAAGTAAGGTTTCAAGCAATTAACCTGGCCGATAATTGCAGACG	Integration in SeSite3
18548	ConR1_Site3	ATATCTCTTAGGCCAGAATATGTTTATTTAACCGTTAAAAGCACTTAGAAGATGACAGGGCCATGATGAGCCGTGATGACCC	
18552	ConLS_Site4	CCAATCAGCGTGTGTTTTATATACCTCTCTTATATAAATTTAAGAAAGAACTGCTTATTCTACTGGCCGATAATTGCAGACG	Integration in SeSite4
18553	ConR1_Site4	TGTTGGTTTTTATAAGCGCATGATGTTGTTGTTGAGAGTCTTAAAGTCAAAATAACACCTGCATGATGAGCCGTGATGACCC	
18981	ConLS_ScEEB1	GGTAGAGAAAGTGGATCGGCGGATTTGAAAGCACAGCGTGGGGAGGATGGTAAATAGAGAACCCTGGCCGATAATTGCAGACG	Integration in ScEEB1
18982	ConR1_ScEEB1	TTTTCCGTATTTTGAAGATTAGCAAAAAGATCAAGATATCAAGTATTTTCATATTTGTCCATGATGAGCCGTGATGACCC	
18985	ConLS_ScEAT1	TGCTGTGTGCGTTGATTTGGGCCGACACGAGAAGAAAGTCCGTTACGTACATCAAGATTACTGGCCGATAATTGCAGACG	Integration in ScEAT1
18986	ConR1_ScEAT1	CCAAGTTCGAGACGTATACAAACTGCAAAAATAAAGGAACCGTGGGAAGAGGGCTTACAACATGATGAGCCGTGATGACCC	
18768	ConR1_SHR-CB-Rv	CGGTGATGGGATACAATCTAGATAAGTTGCGCTGTAGCAGCAAGCTGAATAGCGATGCCATGATGAGCCGTGATGACCC	In vivo assembly SHR CB flank incorporation
13047	ConLS_fw_CB	GCATCGCTATTACGCTTGTCTGTACAGCGCAACTTATCTAGATTGTATCCCATCTGACCCGCCGATAATTGCAGACGAAC	
Diagnostic primers for integration verification			
18514	dg_YCL049C_fw	TCAGTTAGAGTGACAGTTGCG	Integration in SeYCL049C
18515	dg_YCL049C_rv	GTTGTGCGTTTACGTGC	
18519	dg_YCL036W_fw	TCCGTCAGTATTCGAGGC	Integration in SeYCL036W
18520	dg_YCL036W_rv	TCTTGGAACTATCTGCG	
18990	dg_YCL012C_2_fw	GGCTCGAATTTTATTTGAGTGG	Integration in SeYCL012C
18991	dg_YCL012C_2_rv	GGTATCTAAAATGCGTTCAAGG	
18529	dg_YCR051W_fw	CCATTCATGTTTAAAGTTTCGGG	Integration in ScYCR051W
18530	dg_YCR051W_rv	GCTTTTCTTCACTCTACAACG	
18534	dg_YCR087C-A_fw	GAAATACCTCTTCGAAACGTTGAG	Integration in ScYCR087C-A
18535	dg_YCR087C-A_rv	ATGAGTGGACTGGCAGC	
18539	dg_Site1_fw	GGACATGAAAGAGCCAG	Integration in SeSite1
18540	dg_Site1_rv	CTCAAACGCTACAAAGGAAGC	

(continued on next page)

Table 3 (continued)

Number	Primer name	Sequence (5' -> 3')	Purpose
18544	dg_Site2_fw	CGTTGGAAAGAGTTGTTACTTTCCTG	Integration in SeSite2
18545	dg_Site2_rv	GCTTTACACTGATATCAAATAGCC	
18549	dg_Site3_fw	GAGGTCAAATGTTGATAATTAGGAG	Integration in SeSite3
18550	dg_Site3_rv	GCATTTGTAATATTTCCAAGCTGC	
18992	dg_Site4_2_fw	GGAATATGTGACTTCGGGC	Integration in SeSite4
18993	dg_Site4_2_rv	ACCTAATCAACGTGAAGGC	
15944	dg_ScEEB1_fw	AGTGCCGCTTCGAAATCATC	Integration in ScEEB1
15945	dg_ScEEB1_rv	TTCTGCATCTGGTTGCCCTAC	
15950	dg_ScEAT1_fw	CTGGGTTGGAACGAAGTTTG	Integration in ScEAT1
15951	dg_ScEAT1_rv	ACCGTCATGAGTGTAGTCAG	

d at 20 °C while shaking at 200 rpm. Then, 1 mL of the cell suspension was transferred to 20 mL fresh YPD hyg medium in shake flasks and grown for 3 d. Finally, 1 mL cell suspension was transferred to non-selective YPD medium for optimal fluorescent gene expression. *In vivo* assembly efficiency of ymNeogreen and ymScarlet genes in CBS 1483 was analysed by measuring fluorescence levels in the BD FACS Aria™ II SORP Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA) equipped with 355, 445, 488, 561 and 640 nm lasers and a 70 µm nozzle, and operated with filtered FACSFlow™ software (BD Biosciences). The fluorophore ymScarletI was excited by the 561 nm laser and emission was detected through a 582 nm bandpass filter with a bandwidth of 15 nm. The fluorophore ymNeogreen was excited by the 488 nm laser and emission was detected through a 545 nm bandpass filter with a bandwidth of 30 nm. The cytometer performance was evaluated prior to each experiment by running a CST cycle with CS&T Beads (BD Biosciences) and the drop delay for sorting was determined by running an Auto Drop Delay cycle with Accudrop Beads (BD Biosciences). For each sample, 100,000 events were analysed. Cell morphology was analysed by plotting forward scatter (FSC) against side scatter (SSC) and the appropriate cell size was gated. Gated cells were used to determine the fluorescence intensity of the cells. Gating windows for fluorescence intensity were based on the fluorescence of the cells transformed with solely ymNeogreen or ymScarletI as repair fragment for sole integrations. Cells in the gate ymNeogreen⁺ymScarlet⁺ were sorted separately on non-selective YPD plates and grown for 5 d. FACS data was analysed using the Flowing Software version 2.5.1 (Turku Centre for Biotechnology, Finland).

Fermentation in septum flasks

Frozen aliquots of CBS 1483, IMI504-IMI508, IMI510–512, IMI483 and IMI485 were inoculated in 20 mL of YPD media in 50 mL Greiner tubes with air vents at 12 °C shaking at 200 rpm and transferred to precultures in 100 mL YPD in 500 mL shake flasks grown at 12 °C shaking at 200 rpm. Exponentially growing cells were washed and transferred into 100 mL septum bottles containing 60 mL full malt wort at 5.7°P for strains CBS 1483, IMI504-IMI508, IMI510–512 or in full malt wort at 17°P for strains CBS 1483, IMI483 and IMI485 at a starting OD₆₆₀ of 0.2 mL⁻¹. The cultures were incubated at 12 °C and at 200 rpm for five (5.7°P) or eight days (17°P) with regular daily sampling to determine cell density, sugar consumption, ethanol production, esters and ketones concentrations throughout the fermentations.

Analytical techniques

Sugars (glucose, fructose, maltose, maltotriose) and ethanol concentrations were measured using HPLC (Agilent Technologies, 1260 HPLC system; Agilent Technologies, Santa-Clara, CA) equipped with a Bio-Rad HPX-42A column (300 × 7.8 mm, 25 µm) (Bio-Rad, Hercules, CA) and an 1260 Refractive Index Detector as previously described in [11]. Vicinal diketone (diacetyl, 2, 3-pentadione) concentrations were measured using static headspace gas chromatography (GC) in a 7890 A Agilent GC (Agilent Technologies) with an electron capture detector on a

CP-Sil 8 CB (50 m × 530 µm × 1 µm) capillary column as previously described in [15]. Higher alcohols and esters were analyzed using static headspace gas chromatography (GC) with a flame ionization detector FID (Agilent technologies 7890 A) and a DB-WAXetr capillary column (30 m × 320 µm × 1 µm) as described in [11].

Whole genome sequencing

Yeast genomic DNA of transformants was isolated using QIAGEN Genomic-tip 100/G kit (Qiagen, Hilden, Germany). Genomic DNA concentrations were measured with the BR ds DNA kit (Invitrogen, Carlsbad, CA, USA) using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). In-house DNA sequencing was performed using the MiSeq System (Illumina, San Diego, CA, USA). Extracted DNA was mechanically sheared to an aimed average size of 550 bp with the M220 ultrasonicator (Covaris, Wolburn, MA, USA). DNA libraries were prepared using the TruSeq DNA PCR-Free Library Preparation Kit (Illumina) according to the manufacturer's manual. Quantification of the libraries was performed by qPCR using the KAPA Library Quantification Kit for Illumina platforms (Kapa Biosystems, Wilmington, MA, USA) on a Rotor-Gene Q PCR cyclor (Qiagen). The library quality was verified on the TapeStation System 4150 (Agilent). All libraries were normalized to 2 nM before the multiplexing step.

All Illumina sequencing (suppl. Table S1) data are available at NCBI (<https://www.ncbi.nlm.nih.gov/>) under the bioproject accession number PRJNA911296 (study SRP412695). The raw Illumina reads (suppl. Table S1) were aligned against a chromosome-level reference genome of CBS 1483 (NCBI bioproject accession number PRJNA522669, <https://www.ncbi.nlm.nih.gov/>) [16] using the Burrows–Wheeler Alignment tool (BWA) [34] and further processed with SAMtools [35].

Identification of unique landing sites

Identification of unique landing sites on CHRIII of the CBS 1483 was performed using sequence available at NCBI (<https://www.ncbi.nlm.nih.gov/>) as bioproject accession numbers PRJNA522669 (Illumina) and PRJNA522669 (Nanopore). The coding sequences and additionally 800 bp upstream and 300 bp downstream were subtracted from the SeSc-CHRIII sequence. These non-coding sequences of SeScCHRIII were extracted with bedtools (getfasta option) [36]. The remaining sequence was used to generate *k*-mers with *k* = 200 and *k* = 500 with the ara package (<https://github.com/AbeelLab/ara>). The resulting *k*-mers were aligned to the CBS 1483 genome using a MUMmer alignment [37] and eliminated upon a match other than its original location in SeScCHRIII. Next, the unique regions were subjected to manual inspection for presence of other essential DNA sequences and discarded when present. Finally, manual screening of the unique landing sites for other essential DNA regions was performed. Genes with non-essential functions as described in the SGD database (<https://www.yeastgenome.org/>) were selected.

CHRIII configuration analysis

S. pastorianus genomes deposited at NCBI (Bioprojects PRJNA522928 [38], PRJDB4073 [39], PRJNA504476, PRJNA169496) were mapped to the concatenated genome sequences of *S. eubayanus* (CBS 12357) and *S. cerevisiae* (CEN.PK113–7D) using the Burrows-Wheeler Alignment (BWA) tool [34] and further process with SAMtools [35]. Coverage of 500 bp window was calculated across the reference and CNV was determined based on Magnolya [40].

Results

Targeting homoeologous genes in CBS 1483 results in unpredictable loss of heterozygosity

To illustrate the unpredictability of the outcome of CRISPR editing in allo-aneuploidy yeast, individual knockout of genes *ScEEB1* and *ScEAT1* was attempted. *ScEEB1* is carried by *ScCHRXVI* (two copies) and has a homoeolog (*SeEEB1*) on *SeCHRXVI* (two copies). *ScEAT1* is present on *ScCHRVII* (one copy) and its homoeolog *SeEAT1* is on *SeCHRVII* (three copies). Deletion of the two copies of *ScEEB1* in *S. pastorianus* CBS 1483 was performed by co-transforming pUDP168 that expresses a gRNA targeting *ScEEB1* and a repair fragment containing the ymNeogreen

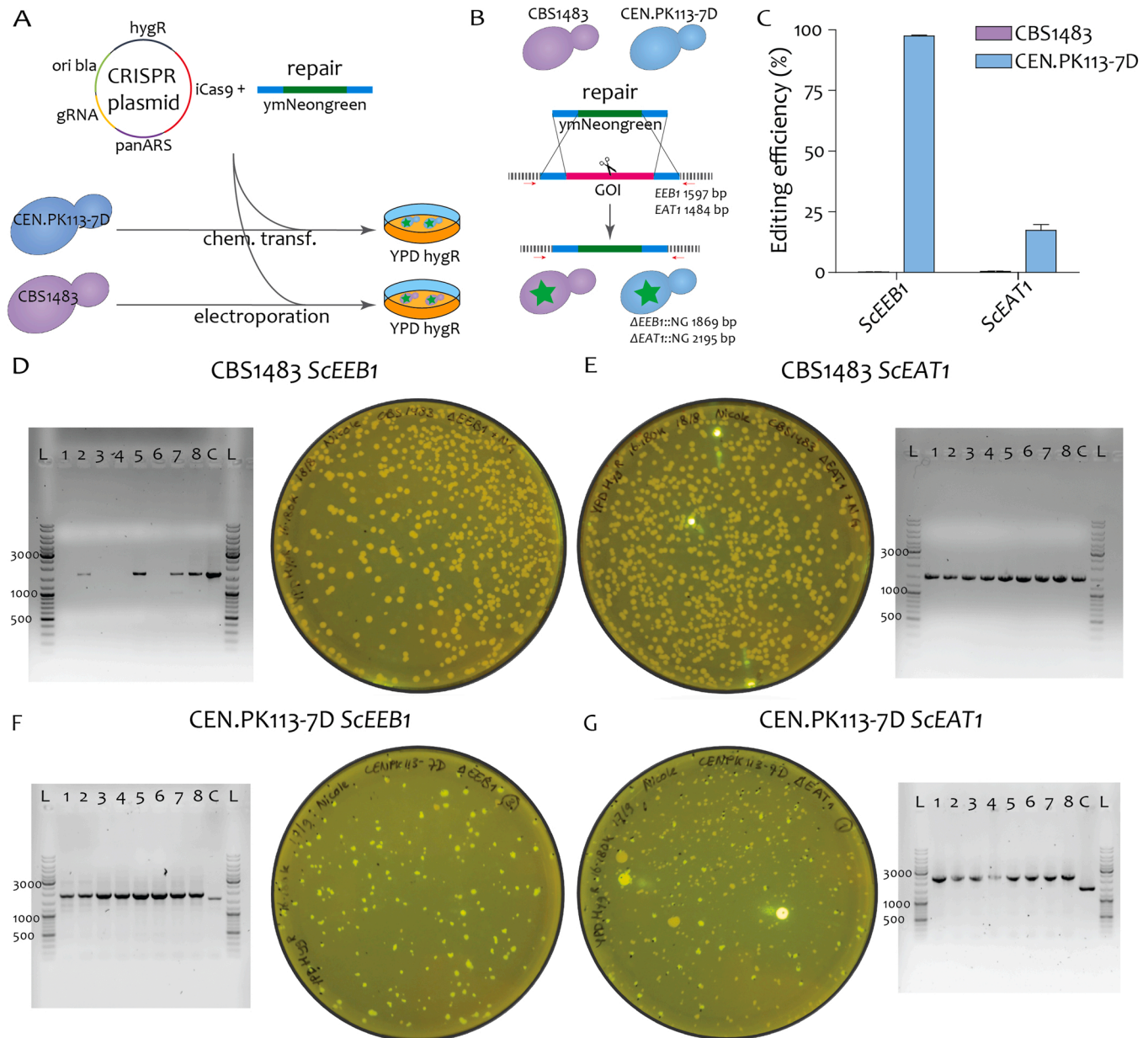


Fig. 1. Genome editing in haploid *S. cerevisiae* CEN.PK113–7D and aneuploidy *S. pastorianus* CBS 1483. A) Schematic representation of the transformation setup and genomic modification. B) Genome editing efficiencies in *S. pastorianus* CBS 1483 (purple bars) and *S. cerevisiae* CEN.PK113–7D (blue bars) targeting the *S. cerevisiae* allele (CBS 1483) or gene (CEN.PK113–7D) *ScEEB1* and *ScEAT1* thereby aiming for ymNeogreen integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations. Transformation plates and genotyping of transformants of ymNeogreen integration in *ScEEB1* in *S. pastorianus* CBS 1483 (C), *ScEEB1* in *S. cerevisiae* CEN.PK113–7D (D), *ScEAT1* in *S. pastorianus* CBS 1483 (E) and *ScEAT1* in *S. cerevisiae* CEN.PK113–7D (F). Integration of ymNeogreen results in green fluorescent colonies. Genotyping confirmed integration of ymNeogreen in *ScEEB1* and *ScEAT1* in *S. cerevisiae* CEN.PK113–7D, but not in *S. pastorianus* CBS 1483.

flanked by 60 bp nucleotides homologous to upstream and downstream the *ScEEB1* open reading frame. Similarly deletion of *ScEAT1* was performed using pUDP172 and a ymNeogreen containing repair targeting the *ScEAT1* locus (Fig. 1A-B). Integration of ymNeogreen at the target site resulted in green fluorescent colonies when excited by blue light (Fig. 1A-B). Despite the previously shown successful editing (>90%) of *SeLV6*, *SeATF1* and *SeATF2* in CBS 1483 with CRISPR-Cas9 [26], integration of the ymNeogreen marker at *ScEEB1* or *ScEAT1* loci yielded 0.19% and 0.48% targeting efficiencies, respectively (Fig. 1C). Even though fluorescent transformants were observed, none of them showed the right genotype (Fig. 1D-E).

To eliminate the possible weak activity of the gRNA to programme Cas9 targeting at both sites, the same two deletions were also performed in the haploid *S. cerevisiae* CEN.PK113–7D. Contrasting with the poor efficiency observed in CBS 1483, all CEN.PK113–7D fluorescent transformants tested harbored the correct deletion (Fig. 1C-F-G). However, we observed a clear difference in targeting efficiency between the *ScEEB1* (100%) and *ScEAT1* (20%) editing events (Fig. 1C). This might have been caused by lower spacer efficiency; even though the spacer design included folding analysis to mitigate occurrence of secondary structures, these events that might hinder target recognition could not be entirely eliminated. Additionally, DNA conformation at the spacer location such as nucleosome positioning, could also affect the editing efficiency; however no further work to elucidate this phenomenon was attempted.

Assuming that the Cas9 guided by the respective gRNAs introduced the DSB successfully, the remaining question was how does *S. pastorianus* repair the DSB event that, if not repaired, is lethal for the cell. To elucidate this mechanism, four non fluorescent transformants from *ScEEB1* deletion transformation were randomly selected and subjected to whole genome sequencing (WGS).

Sequencing analysis confirmed that transformants had failed to incorporate the supplied repair fragment, but instead repaired the DSB with the homoeologous *S. eubayanus* CHR XVI as template for HDR, resulting in loss of heterozygosity (Fig. 2, suppl. Fig. S1, suppl. Table S1). Each transformant exhibited a different pattern: colony 1 (IMK1062), colony 4 (IMK1064) and colony 3 (IMK1063) were repaired by a 5 kbp, 10 kbp and 26-kbp region surrounding the *ScEEB1* gene, respectively, while in colony 6 (IMK1065) over 700 kbp from the *ScCHR XVI* was replaced by the homologous sequence from the *SeCHR XVI* sequence and only the subtelomeric regions of *ScCHR XVI* were retained (Fig. 2, suppl. Fig. S1, suppl. Table S1). Therefore, loss of heterozygosity resulting from preferential usage of the homoeologous chromosome is an undesired side-effect of Cas9-mediated genome editing and has a major impact on the editing specificity and efficiency in allo-aneuploid yeasts, including *S. pastorianus* CBS 1483.

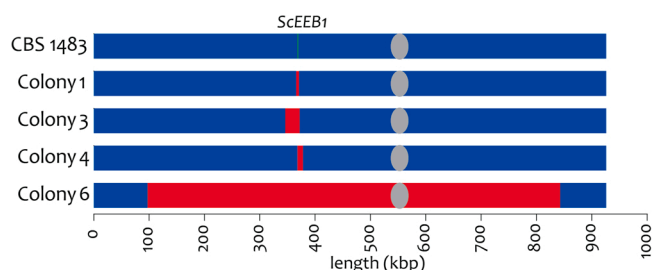


Fig. 2. Whole genome sequencing reveals that CRISPR-Cas9 editing in the allo-aneuploid yeast *S. pastorianus* CBS 1483 aiming to integrate ymNeogreen in *ScEEB1* on *ScCHR XVI* results in loss of heterozygosity in all four colonies tested. The red and blue color indicate chromosome assigned as *S. eubayanus* and *S. cerevisiae* respectively.

A systematic strategy to identify unique landing sites for CRISPR-Cas9 targeting

Genome editing with CRISPR-Cas9 allows gene deletions, gene integrations and gene modifications to be made. In the case of gene deletions and gene modifications, the user is restricted to the location of the gene of interest in the genome. On the other hand, additional genes can be integrated at any user-chosen genomic location. Therefore, identification of chromosomal regions (landing sites) devoid of homoeologous regions for gene integrations with CRISPR-Cas9 in the allo-aneuploid yeast strain *S. pastorianus* CBS 1483 would be of a prime importance. Landing sites should meet the following criteria: (i) the frequency for LOH is low, (ii) the gRNA targets the protospacer with high efficiency, and (iii) gene integration does not result in undesired changes in the cell's physiology.

To prevent unwanted repairs that would use the homoeologous chromosome, the search for landing sites was first limited to the chimeric *SeScCHR III*, that is composed of two-thirds from the *S. eubayanus* parent and one-third from *S. cerevisiae*, and is characterized by the absence of homoeologous sequence [15,16]. First, a systematic approach to finding suitable integration sites on *SeScCHR III* was developed. Since modification at a landing site should remain metabolically neutral, i.e. should not impact yeast physiology, coding sequences including 800 bp upstream and 300 bp downstream covering the promoter and terminator regions were subtracted from the *SeScCHR III* sequence. This thorough *in silico* analysis revealed a set of 23 non-coding regions (total of 34.7 kbp) ranging from 30 bp to more than 6 kbp (Fig. 3, suppl. Table S2). These non-coding sequences were used to generate *k*-mers of 100, 150, 200, 300 and 500 nucleotides which were aligned to CBS 1483 genome using MUMmer [37]. *K*-mers with small, partial alignments to other chromosomes were discarded to avoid selection of sequences containing homology with other parts of the genome and only *k*-mers that returned one hit located on *SeScCHR III* were selected. This resulted in a total of 283 and 50 unique *k*-mers for respectively *k* = 200 and *k* = 500, residing in a total of 26 regions (total 22.4 kbp, average length of 863 bp) and 17 regions (total 23.9 kbp, average length of 1403 bp), respectively. Since unique *k*-mers could be adjacent and overlap one another, the 17 unique regions of the *k* = 500 output were identified spanning lengths from 198 to 3213 bp. These 17 unique regions were manually checked for the presence of other essential DNA regions, such as tRNAs, telomeres, centromeres and autonomous replication sequences. Out of the 13 regions that passed the final check, four landing sites distributed over *SeScCHR III*, referred to as Sites 1, 2, 3 and 4, were chosen to investigate their potential use for genomic integrations in CBS 1483. Secondly, a manual screening of *SeScCHR III* for non-essential genes was deployed to find landing sites located in gene encoding regions. The search resulted in 13 genes suitable as landing sites (suppl. Table S3) of which the following five genes distributed over the chromosome were selected: *SeYCL049C*, *SeYCL036W*, *SeYCL012C*, *ScYCR051W* and *ScYCR087C-A*. In total, nine genes and sites were retained as potential landing sites on CBS 1483 *SeScCHR III* (Table 4, Fig. 3).

CRISPR-Cas9 targeting of unique landing sites allows efficient gene integration

To evaluate the landing sites for genomic integrations, gRNAs for all landing sites were designed considering GC-content, secondary structure, off-targets and allelic variation that may occur within the four copies of the CBS 1483 chimeric *SeScCHR III* (suppl. Table S4). To evaluate the CRISPR-editing in the 9 selected landing sites, the CRISPR-plasmid co-expressing *Streptococcus pyogenes* iCas9^{D147Y,P411T} and the respective gRNA [30] were co-transformed with the ymNeogreen repair fragment, allowing visual screening of edited colonies, into CBS 1483.

Targeting the landing sites *SeYCL049C*, *SeYCL036W*, *SeYCL012C*,

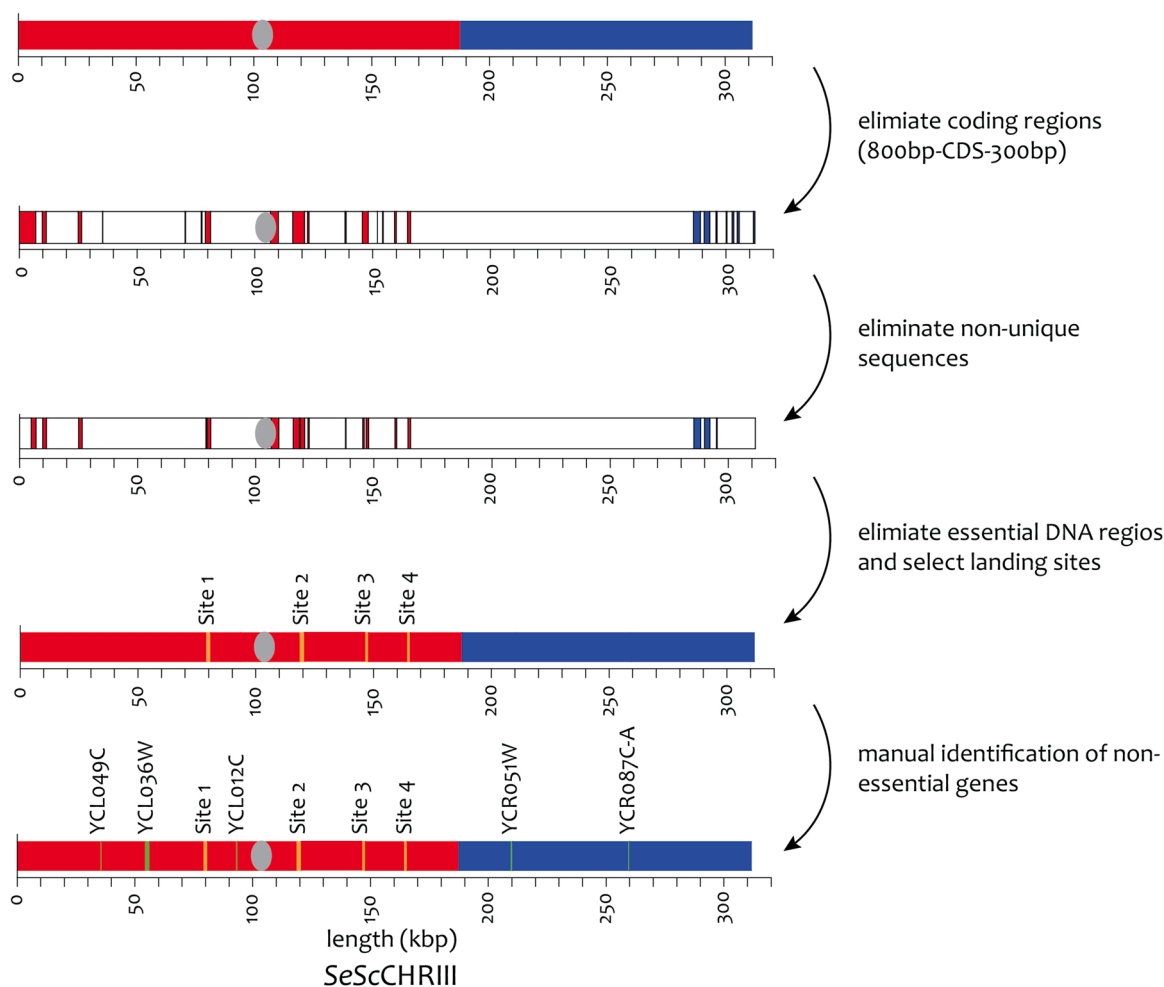


Fig. 3. Strategy to identify non-coding unique DNA sequences in CBS 1483 *SeScCHRIII*. In the first step, non-coding regions were obtained by subtracting the coding sequences plus 800 bp upstream and 300 bp downstream covering for promoter and terminator regions. In the second step, the unique regions were obtained by selecting unique *k*-mers when aligning to the CBS 1483 genome and the non-unique sequences were eliminated. In the next step, the non-coding unique sequences were checked for the presence of essential DNA regions. Additionally, potential landing sites identified by manual screening of non-essential genes were selected, resulting in a total of nine landing sites.

Table 4

Overview of the selected landing sites on *SeScCHRIII* in *S. pastorianus* CBS 1483.

Landing site	Start coordinate	Stop coordinate	Strand	Size (bp)
YCL049C	35643	35915	-	884
YCL036W	54552	56237	+	1685
Site 1	79419	80823	n.a.	1404
YCL012C	93151	93617	-	466
Site 2	118822	120628	n.a.	1806
Site 3	146704	147799	n.a.	1095
Site 4	164540	165542	n.a.	1002
YCR051W	209678	210346	+	668
YCR087C-A	259551	259985	-	434

n.a. Not Applicable

ScYCR051W, *ScYCR087C-A*, *SeSite2*, *SeSite3* and *SeSite4* resulted in high editing efficiencies, which is reflected in the number of green fluorescent colonies on the transformation plates (Fig. 4). Genotyping of 10 colonies for each landing site showed 100% correct ymNeogreen integration in *SeYCL049C*, *SeYCL012C*, *ScYCR051W*, *ScYCR087C-A*, *SeSite2*, *SeSite3* and *SeSite4* (suppl. Fig. S2). Genotyping of *SeYCL036W* showed that 11 out of the 12 assessed colonies were correctly edited and showed correct integration of the ymNeogreen expression cassette (suppl. Fig. S3). Only integration at *SeSite1* did not result in high editing efficiencies, possibly due to the low gRNA efficiency (Fig. 4). Thus, 8 out

of 9 identified landing sites showed high potential for standard genomic integrations in *S. pastorianus* CBS 1483 as they were targeted with high efficiency and do not cause loss of heterozygosity.

Some of the landing sites can be used in multiple S. pastorianus strains: the example of Weihenstephan 34/70

The use of these integration sites is not just restricted to the CBS 1483 strain. Genome-wide analysis of different *S. pastorianus* genomes deposited at NCBI (<https://www.ncbi.nlm.nih.gov/>), revealed that the large majority of the strains (45 out of 47 strains) harbors the chimeric *SeScCHRIII* in various copy numbers, either as sole *CHRIII* configuration (two strains) or in combination with *ScCHRIII* (39 strains) or *SeCHRIII* (four strains) (Fig. 5A). In any configuration, the entire *SeScCHRIII*, if present alone as in CBS1483 or part of it, is unique. This is exemplified by the *S. pastorianus* WS 34/70 strain, which counts 3 copies of the chimeric *SeScCHRIII* and one full length copy of the *ScCHRIII* (Fig. 5B). Whereas the sequence similarity of the shared *ScCHRIII* part is close to 100%, the sequence homology between the *SeCHRIII* part and the *ScCHRIII* part is only 80–85%. In this genome configuration, this means that only the distal *S. cerevisiae* sequence of *SeScCHRIII* including the *ScYCR051W* and *ScYCR087C-A* sites could lead to efficient editing. To test this hypothesis, the WS 34/70 strain was transformed with the gRNA expressing plasmids targeting the 8 most efficient landing sites

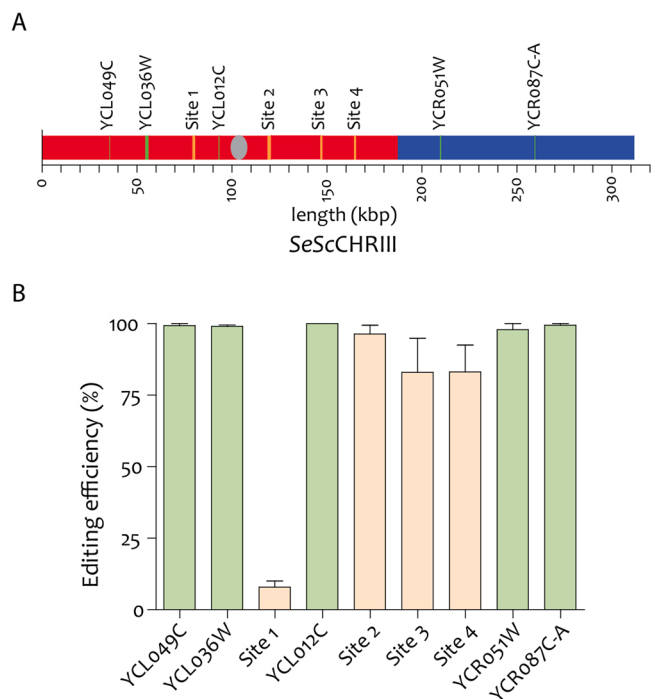


Fig. 4. A) Overview of the identified landing sites in CBS 1483. B) Genome editing efficiencies in *S. pastorianus* CBS 1483 targeting the identified landing sites on *ScSeCHRIII* thereby aiming for *ymNeongreen* integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations.

previously identified and their respective repair DNA containing the fluorescent marker. The integration sites lacking homoeologous sequences, *ScYCR051W* or *ScYCR087C-A*, showed, as anticipated, a high targeting efficiency exceeding 50% (Fig. 5B). For the proximal landing sites *SeYCL049C*, *SeYCL036W*, *SeYCL012C* and *SeSite2*, there was a significant drop in efficiency (<50%). Remarkably, the efficiency of the editing at *SeSite3* and *SeSite4* was to a lesser extent not hampered by the presence of the *S. cerevisiae* sequence, contrasting with results at other chromosomal sites indicating that other genetic factors might play a role.

Genomic integrations at landing sites do not result in changes in physiology and brewing characteristics

One of the criteria for a good landing site is that integration of DNA at this location should not have a physiological effect on the cell compared to the parental CBS 1483 strain. To investigate potential physiological effect on genomic integrations, the strains CBS 1483 and IMI504–8 and IMI510–512 containing *ymNeongreen* in their respective landing sites, were cultivated in septum flasks under brewing conditions (full malt wort at 5.7° P and at 12 °C). Deviations in growth, sugar consumption, flavor molecule production and ethanol production were evaluated relative to the parental strain CBS 1483 (Fig. 6). Early in the fermentation, the deviations in produced metabolites (e.g. glycerol ($0.013 \pm 0.033 \text{ g L}^{-1}$ for CBS 1483 and $0.000 \pm 0.000 \text{ g L}^{-1}$ for all other strains, compared to $> 0.700 \text{ g L}^{-1}$ respective end concentrations) and ethyl acetate ($0.147 \pm 0.127 \text{ mg L}^{-1}$ for CBS 1483 and $0.000 \pm 0.000 \text{ mg L}^{-1}$ for six other strains, compared to $> 1.100 \text{ mg L}^{-1}$ respective end concentrations)) can have large effects on the fold change, but represent only a very small fraction of the final concentrations. Integration in the landing sites *SeYCL049C*, *SeYCL036W*, *SeYCL012C*, *ScYCR051W*, *ScYCR087C-A*, *SeSite2* and *SeSite3* did not have a significant effect on the physiological behaviour of the strain and

are therefore suited for gene integrations. In contrast, IMI512 (site4: *ymNeongreen*) showed slower growth as well as slower sugar consumption and ethanol production compared to the parental strain CBS 1483. Also, the production of vicinal diketones and esters that contribute to beer flavor was lower for ethyl acetate, isoamyl alcohol, isoamyl ester and isobutanol, whereas the production of ethyl butyrate was higher compared to CBS 1483 after 6 days of cultivation. These observations would suggest that *SeSite4* would be less suitable for integration of genes of interest. However, although genotyping of the *ymNeongreen* integration at the *SeSite4* predicted a correct integration, it could not be excluded that other unintended modifications occurred during the transformation (e.g. alteration of chromosome copy number) since the tested transformant was not whole genome sequenced [19].

Integration of acetolactate decarboxylase to eliminate diacetyl production

To demonstrate the reliability of the selected sites, lager brewing yeast strains with reduced diacetyl production were constructed. The vicinal diketone diacetyl is one of the main off-flavors in lager-style beers. The lagering period after the fermentation is required for the reduction of diacetyl and has a major impact on the time and energy of the brewing process. Diacetyl is formed in the supernatant by spontaneous decarboxylation from excreted α -acetolactate, an intermediate of valine and leucine biosynthesis. During the lagering, the brewing yeast reduces diacetyl sequentially to acetoin and 2,3 butanediol [41,42].

To prevent α -acetolactate leakage, an α -acetolactate decarboxylase (*AldC*), that catalyzes the decarboxylation of α -acetolactate to acetoin, can be expressed. α -*aldC* genes of prokaryotic origin have already been successfully expressed in brewing yeasts, including *S. cerevisiae* [43,44], *S. uvarum* [44,45], *S. carlsbergensis* [46] and *S. pastorianus* [47] have shown great reduction in diacetyl formation resulting in significant shortening of the lagering. Two different α -*aldC* genes were chosen, originating from *Brevibacillus brevis* (*BbaldC*) and *Leuconostoc lactis* (*LlaldC*). To ensure high gene expression levels, the genes were placed under control of the constitutive *ScPGK1* promoter [48] and the *ScADH1* terminator. Linear fragments containing the expression cassettes with 60 bp homologous flanks were co-transformed with the CRISPR-plasmid pUDP273 targeting the landing site YCR087C-A into CBS 1483. The transformation efficiency, calculated based on the percentage of positive colonies verified by diagnostic PCR, was 100% and 79% for *BbaldC* and *LlaldC*, respectively (suppl. Table S6). The correctly edited transformants were named IMI483 (*BbaldC*) and IMI485 (*LlaldC*).

The strains IMI483 (*BbaldC*) and IMI485 (*LlaldC*), were assessed for diacetyl production in septum flask cultivation on 17 P full malt wort at 12 °C and compared to CBS 1483. In agreement with previous results [42–46,49–51], heterologous expression of an α -*aldC* gene in CBS 1483 likely eliminated the diffusion of α -acetolactate into the extracellular space preventing formation of diacetyl and therefore the need of its reduction (Fig. 7). Nonetheless, the growth of IMI485 was hampered compared to the parental strain CBS 1483, caused by loss of the ability to consume maltotriose (suppl. Fig. S4). As previously reported for *SeSite4*, despite correct integration, specific transformants might exhibit altered performances probably caused by unintended genetic alterations. Although, this case showed that the landing sites identified for *S. pastorianus* CBS 1483 were suitable for efficient and reliable engineering requiring CRISPR-Cas9 guided integration, a more extensive geno- and phenotyping characterization would be required to select engineered strains retaining all essential brewing traits.

Successful genomic integration through in vivo assembly into *ScSeCHRIII* landing sites

One of the startling genetic characteristics of *S. cerevisiae* is its ability to recombine multiple linear DNA fragments at a single chromosomal locus. This is often referred to as *in vivo* DNA assembly. We demonstrated that elimination of competing mechanisms, using the homoeologous

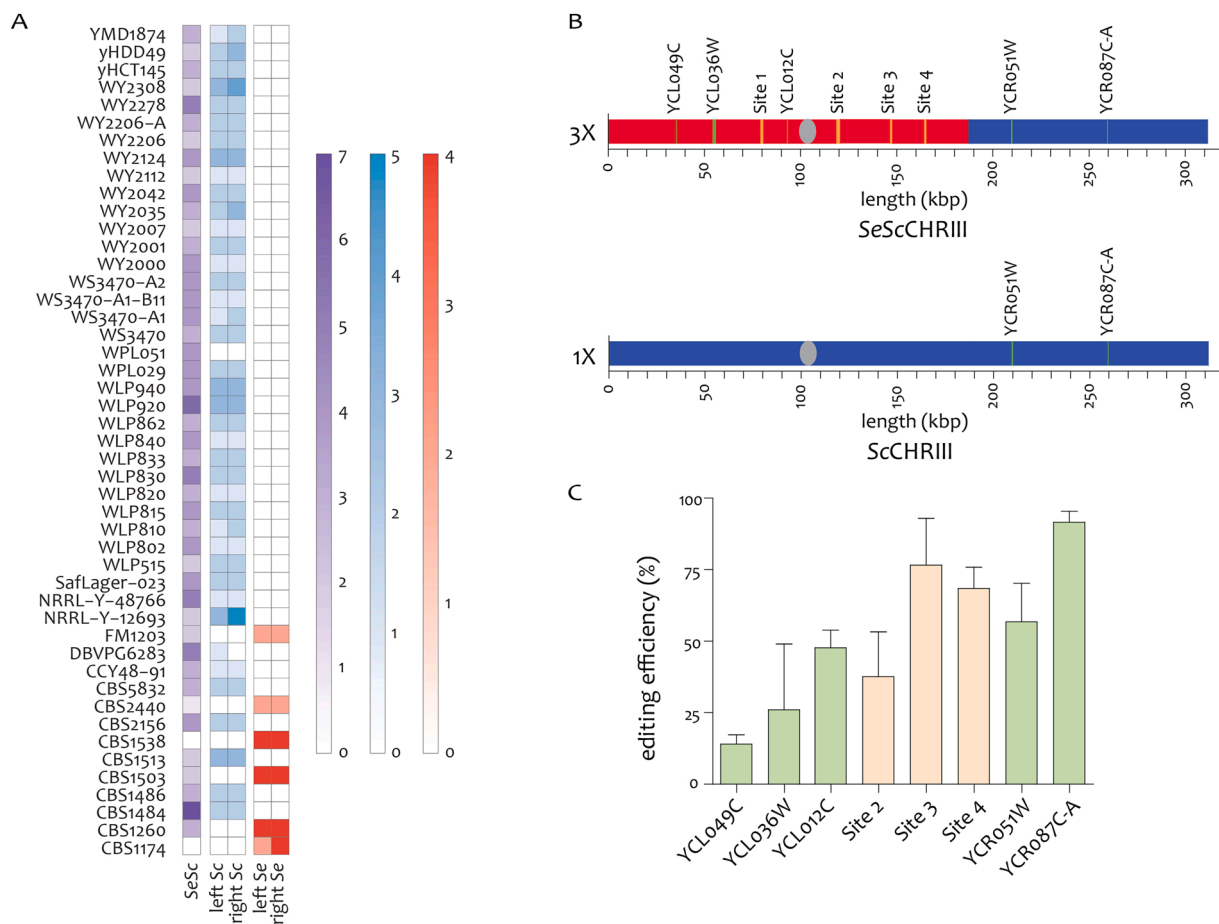


Fig. 5. A) Overview of CHRIII genetic configurations in 47 different *S. pastorianus* strains. B) Chromosome III configurations in *S. pastorianus* WS 34/70. In comparison to CBS 1483, WS 34/70 has an additional ScCHRIII and one less hybrid SeScCHRIII. C) Genome editing efficiencies in *S. pastorianus* WS 34/70 targeting the identified landing sites thereby aiming for ymNeogreen integration. Editing efficiencies were calculated based on number of green fluorescent colonies over the total number of colonies on the transformation plates. Values are derived from three individual transformations.

chromosome as template, improved targeting efficiency of repair fragment integration at chosen chromosomal sites. To investigate the possibility to integrate two fragments simultaneously by HDR, *S. pastorianus* CBS 1483 was co-transformed with pUDP269, a plasmid that expresses a gRNA targeting the SeYCL049C landing site and two DNA repair fragments. The first DNA fragment consisted of the ymNeogreen expression cassette that was flanked by 60 bp homologous region upstream to the SeYCL049C genomic locus and 60 bp SHR sequences [52], whereas the second fragment encoded the ymScarletI gene flanked by 60 bp SHR sequence complementary to that of the first fragment and 60 bp homologous flanks downstream the SeYCL049C landing site. The use of fluorescent genes for the integration allowed for screening of the transformed cell population by Fluorescence-Activated Cell Sorting. Screening of the transformant population revealed that $17.67 \pm 1.98\%$ of the transformed cells harbored both fluorescent markers (suppl. Fig. S5). To confirm that the fluorescence was well derived from single and not aggregated cells, for this, single cells from the ymNeogreen⁺ ymScarletI⁺ gated population were sorted on non-selective medium (suppl. Fig. S5). Cultivation of the sorted single cells and subsequent flow cytometry analysis of 12 clones confirmed co-occurrence of both ymNeogreen and ymScarletI fluorescence (suppl. Fig. S6). Genotyping revealed, in all 12 screened colonies, correct integration of both genes in the SeYCL049C landing site (Fig. 8). These results showed that genomic integration of *in vivo* assembled DNA fragments is also possible in the interspecific hybrid *S. pastorianus*.

Discussion

CRISPR-mediated gene editing in diploid and more generally polyploid heterozygous yeasts is drastically impaired when not all chromosomes are simultaneously targeted by a programmed CRISPR endonuclease. In such conditions, the still intact homologous chromosome is used as preferential template to fix the double strand break by homology-directed repair. This resolution of the introduced cut can result in chromosome recombinations, which can lead to loss of heterozygosity [20,53,54]. LOH competes with an intended gene-editing event and results in reduced editing efficiencies and possibly in extensive genetic changes. In this study, it was demonstrated that this mechanism could also occur between homoeologous chromosomes in the interspecific hybrid yeast *S. pastorianus*. While in diploid yeasts the problem might be restricted to a few heterozygous locations, and easily solved by designing guide-RNA targeting all chromosomes, in *S. pastorianus*, an interspecific hybrid that retained the quasi complete parental genomes, the problem is extended to every single nucleotide since any edit on the chromosome of one of the sub-genome e.g. *S. cerevisiae*, can be repaired with its homoeologous counterpart e.g. *S. eubayanus*. As observed in this study, whether repaired by the donor repair DNA or by the homoeologous chromosome, the resulting chromosomal locus was converted identically in all copies, suggesting that the first repaired site preferentially converts the other. This unpredictability, unfortunately, affects the progress of functional genetic studies in hybrid, industrial yeasts, since the introduction of accurate genetic alterations would have to rely on HDR without introduction of a DSB

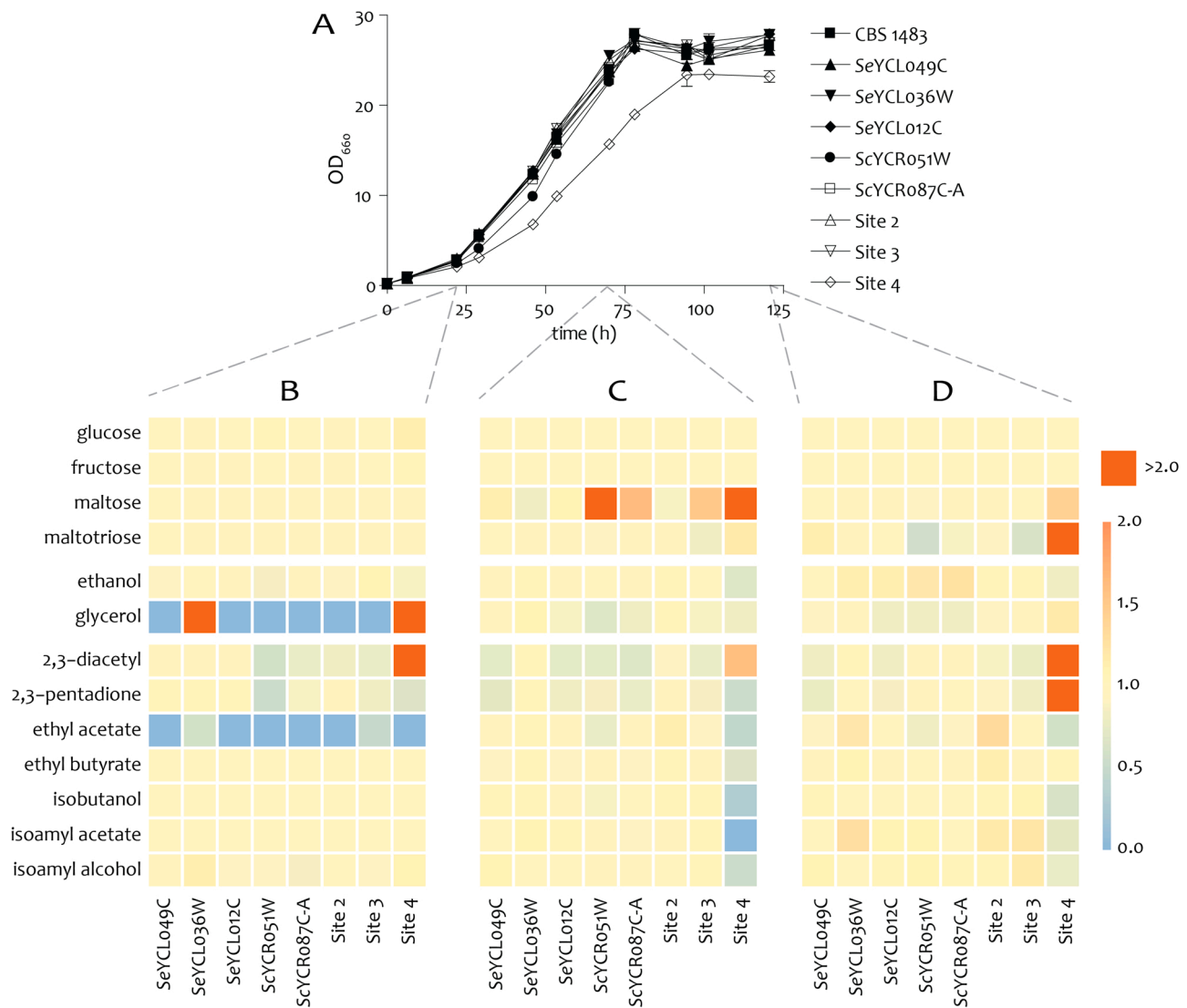


Fig. 6. Physiological characterization of landing site disruptions under brewing conditions. A) Growth curves as function of OD₆₆₀nm of the *S. pastorianus* strain CBS1483 and derived strains IMI504 (Δ SeYCL049C), IMI505 (Δ SeYCL036W), IMI506 (Δ SeYCL012C), IMI507 (Δ ScYCR051W), IMI508 (Δ ScYCR087C-A), IMI509 (Δ SeSite1), IMI510 (Δ SeSite2), IMI511 (Δ SeSite3), IMI512 (Δ SeSite4) harboring a ymNeogreen expression cassettes inserted at single landing sites grown in septum flasks under brewing conditions with full malt wort (5.7° P) at 12 °C. The values represent averages \pm mean deviations of data obtained from independent triplicate cultures. At B) t₁₉, C) t₆₈ and D) t₁₁₆ hours supernatant samples were analysed for extracellular sugars (glucose, fructose, maltose, maltotriose), ethanol, glycerol, vicinal diketones (diacetyl, 2, 3-pentadione), higher alcohols (isoamyl alcohol, isobutanol) and ethyl (ethyl acetate, ethyl butyrate) and acetate (isoamyl acetate) esters. Concentrations relative to the reference strain CBS 1483 are represented in heatmaps and fold-change in concentrations are color-coded according to the scale provided right to heatmap D).

[55,56], methods that require the use of selectable markers and are iterative in nature, since it can replace one allele at the time (e.g. deletion of a gene carried by a trisomic chromosome will require three rounds of transformations) [57,58]. Therefore, improving the predictability of the editing event is critical to enable efficient gene deletion or *in vivo* site-directed mutagenesis.

Genome-wide analysis of different *S. pastorianus* genomes deposited at NCBI (<https://www.ncbi.nlm.nih.gov/>) showed that chimeric *SeSc*-CHRIII chromosome was nearly always present (found in 45 out of the 47 *S. pastorianus* genomes analysed (Fig. 5A)) as sole CHRIII or in combination with either a *Se*CHRII or *Sc*CHRIII version. In any configuration, the entire CHRIII or part of it is unique. In contrast to targeted sites on other chromosomes, the targeting efficiency of the site on *SeSc*CHRIII region devoid of homoeologous sequences was high and reached up to 100% in some instances. Refuting previous statements, these results demonstrated that homologous recombination was not impaired in *S. pastorianus* but was instead masked. It could also be

excluded that genome complexity, increased ploidy and chromosome copy number were also not hindering efficient repair of DSB, as *S. pastorianus* CBS 1483 could efficiently repair tetrasomic *SeSc*CHRIII. CRISPR-Cas9 mediated *in vivo* recombination of two DNA fragments further confirmed that, at selected loci, *S. pastorianus* could perform elaborated homology directed repair with high efficiency. Improving the targeting efficiency in lager brewing yeasts, provides new opportunities to accurately and time-effectively engineer *S. pastorianus* strains with new characteristics. Such strategies could swiftly and efficiently improve a wide range of phenotypic traits and fermentation characteristics, including wort sugar utilization, fermentation rate and energetic performance [6,11], reduction of off-flavor [47,58] and balanced flavor profiles and, moreover, engineering of novel flavors [59,60].

It remains however important to evaluate the targeted integration on the genetic level and screen multiple colonies on phenotype to avoid loss of relevant characteristics resulting from secondary effects. It is indeed known that transformation procedures are mutagenic and in such

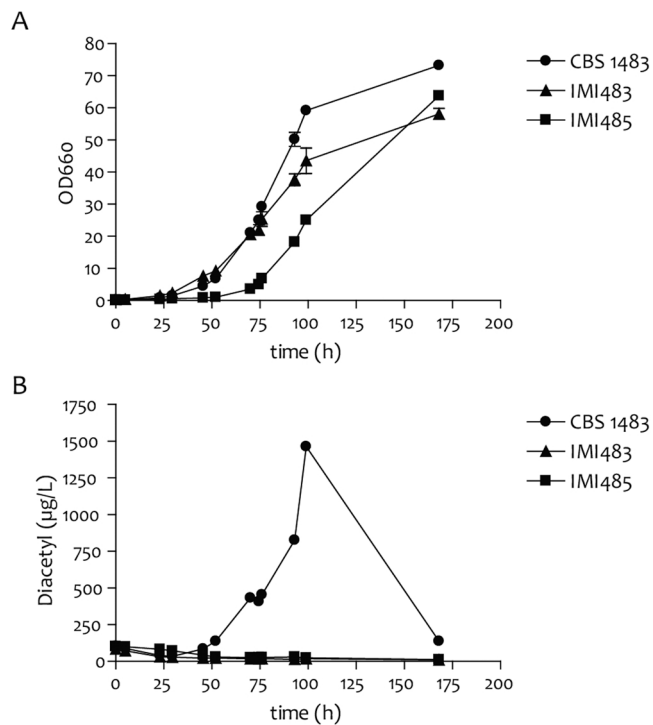


Fig. 7. Performance of CBS 1483 strains expressing different prokaryotic *aldC* genes. A) Growth curve and B) diacetyl concentration of CBS 1483 (●), IMI483 (expressing *BbaldC*, ▲) and IMI485 (expressing *LlaldC*, ■) grown on full malt wort (17 °P, 12 °C). The values represent averages ± mean deviations of data obtained from independent triplicate cultures.

aneuploid organisms may lead to chromosome copy number variations that could impact other phenotypic traits unintentionally [15,19]. To completely eliminate this possibility, systematic genome sequencing of engineered strains could be implemented.

Even though *S. pastorianus* is the most industrially used interspecific hybrid between *Saccharomyces* species yeasts, numerous independently formed natural hybrids between *Saccharomyces* species (e.g. *S. cerevisiae* X *S. kudriavzevii*, *S. cerevisiae* X *S. eubayanus* and *S. cerevisiae* X *S. kudriavzevii* X *S. eubayanus*) have been isolated from wine, beer, or cider fermentations [61–66]. Natural interspecific hybrids are not limited to the *Saccharomyces* species group. Several examples have also been described in *Zygosaccharomyces* [67] and *Millerozyma* [68]. Often, these hybrids exhibit better characteristics in fermentation processes than the parental strains, as this heterosis provides a competitive advantage by enabling transgressive phenotypes in man-made environments, and also drives adaptation and fungal evolution. The guiding principles presented in this study should also be helpful to dissecting the genetic background of heterosis in yeast hybrids other than *S. pastorianus* and contribute to engineering these yeasts in the future.

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CRediT authorship contribution statement

JMGD and NXB designed experiments. MK executed the diacetyl study, NXB executed all other experiments. NXB and MvdB performed bioinformatics analysis. NXB and JMGD supervised the study. NXB and JMGD wrote the manuscript. All authors read and approved the final

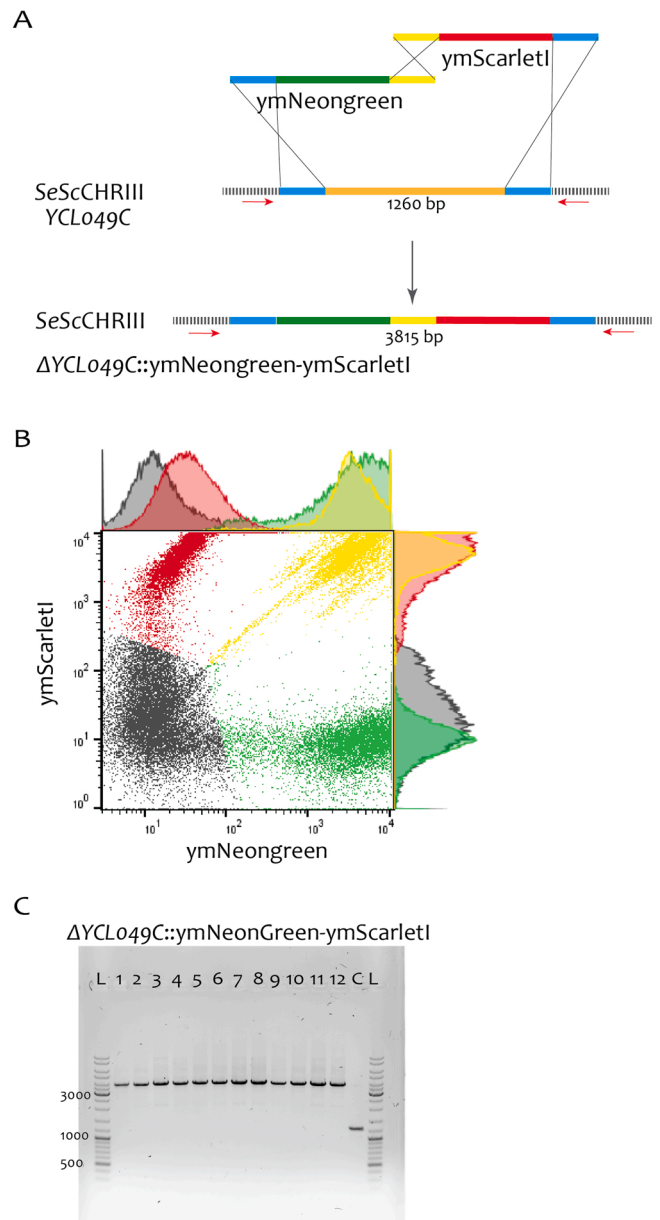


Fig. 8. Genomic integration into landing site *SeYCL049C* of two in vivo assembled DNA fragments expressing ymNeogreen and ScarletI. A) Schematic overview of the genomic integration strategy for in vivo assembly of ymNeogreen and ScarletI DNA fragments. B) Fluorescent profile the transformed population when aiming for integration of two fluorescent expression cassettes. The fluorescence corresponding to ymNeogreen and ymScarlet is plotted for 100,000 events. Flow cytometry was performed for three biological replicates; one representative replicate is shown. C) Genotyping of the sorted ymNeogreen⁺Scarlet⁺ single colony isolates.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data underlying graphs and figures found in this manuscript are deposited at the 4TU research dat center (<https://data.4tu.nl/>) and

available through the doi: 10.4121/21648329.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.04.001.

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