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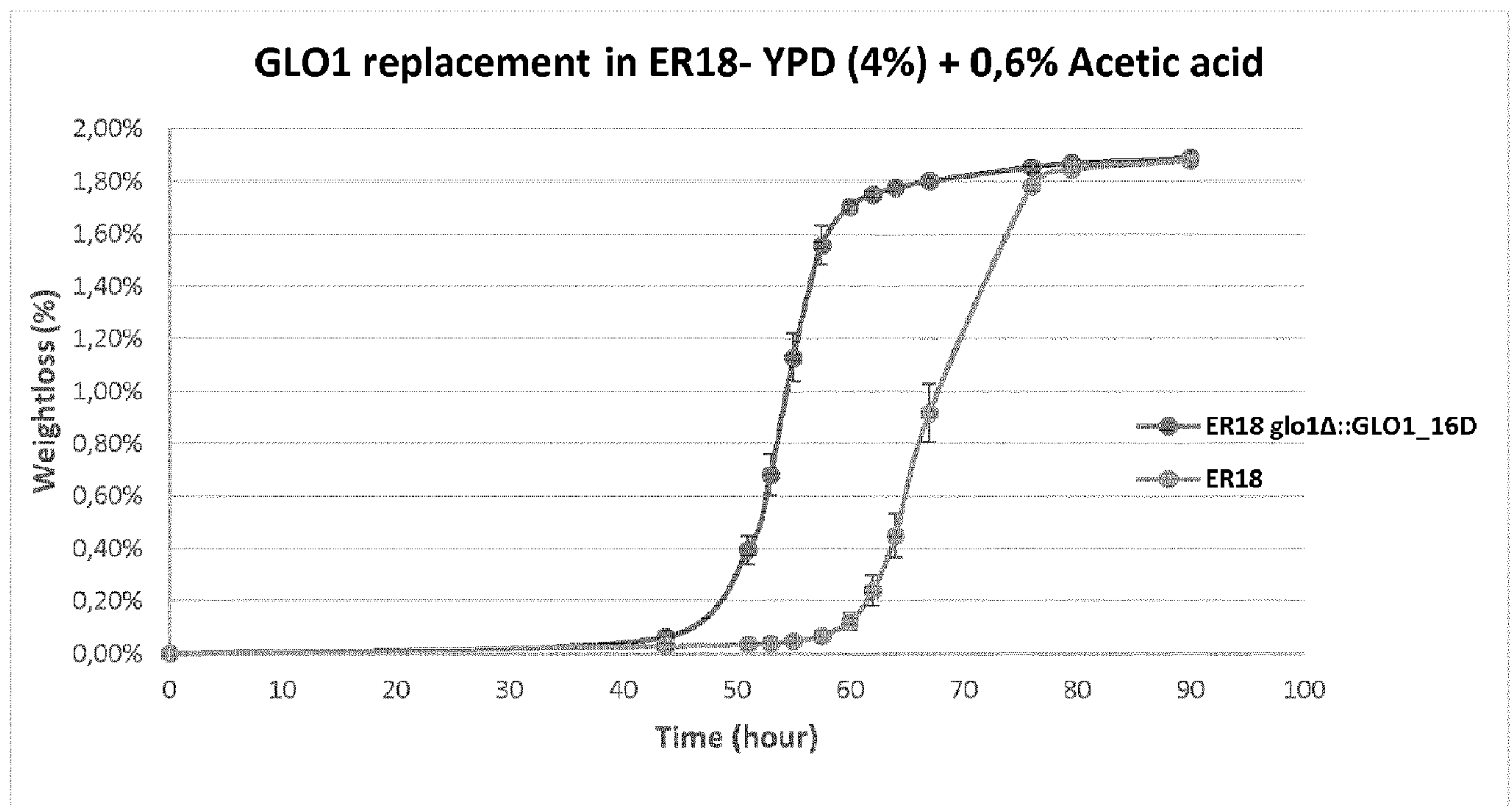
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(54) **Titre : GENES CAUSAUX CONFERANT UNE TOLERANCE A L'ACIDE ACETIQUE CHEZ LA LEVURE**

(54) **Title: CAUSATIVE GENES CONFERRING ACETIC ACID TOLERANCE IN YEAST**

Figure 3



(57) **Abrégé/Abstract:**

The present invention relates to the use of GLO1 to modulate acetic acid tolerance in yeast. More specifically, it relates to the use of a specific GLO1 allele to confer tolerance to acetic acid, and to improve the fermentation performance of yeast in the presence of acetic acid.

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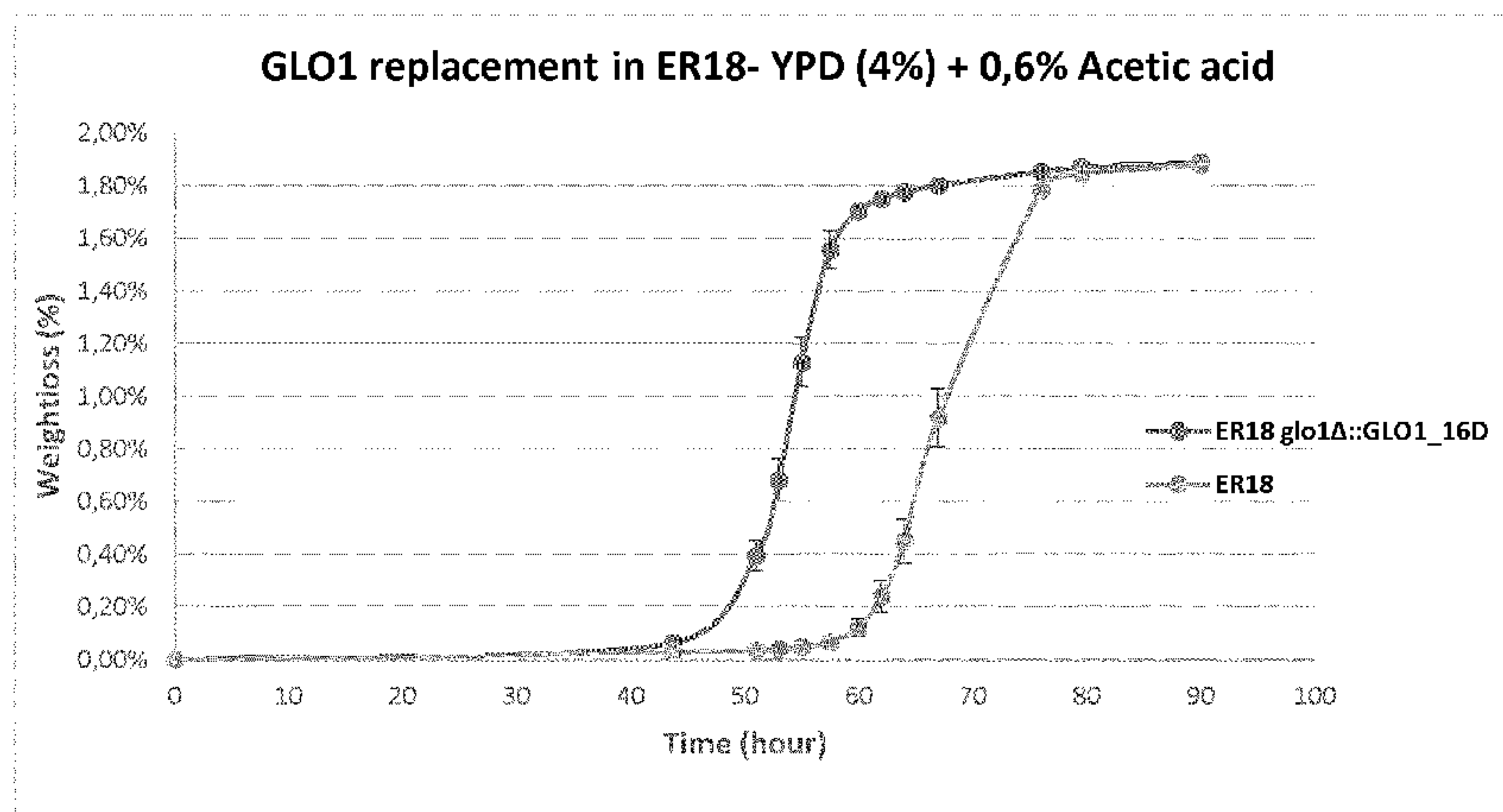
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(54) Title: CAUSATIVE GENES CONFERRING ACETIC ACID TOLERANCE IN YEAST

Figure 3



(57) Abstract: The present invention relates to the use of *GLO1* to modulate acetic acid tolerance in yeast. More specifically, it relates to the use of a specific *GLO1* allele to confer tolerance to acetic acid, and to improve the fermentation performance of yeast in the presence of acetic acid.

Causative genes conferring acetic acid tolerance in yeast

The present invention relates to the use of *GLO1* to modulate acetic acid tolerance in yeast. More specifically, it relates to the use of a specific *GLO1* allele to confer tolerance to acetic acid, and to improve the fermentation performance of yeast in the presence of acetic acid.

5 Hydrolysates of lignocellulose are an interesting source for the production of bioethanol. However, one of the problems is the presence of toxic compounds such as acetic acid, furfural and lignin derivatives. Resistance against these inhibitors is essential for an efficient bioethanol production (Olsson and Hahn-Hägerdal, 1993). Especially acetic acid is known to have an inhibitory effect (Limtong et al., 2000). However, although overexpression of a single
10 gene may improve acetic acid tolerance (Tanaka et al., 2012), it is important to understand the interplay of genes, proteins and other components that determine the physiological properties of a microorganism.

In the past, research focused indeed primarily on the identification of single alleles or genetic loci that are involved in physiological traits (Glazier et al., 2002). However, in contrast to
15 Mendelian traits (traits that are caused by one single locus), quantitative traits are caused by multiple genetic loci, which makes the unraveling of these complex traits rather difficult (Steinmetz et al., 2002). In addition, the genetic mapping of quantitative trait loci (QTL) is hampered by genetic heterogeneity, variable phenotypic contributions of each QTL, epistasis and gene-environment interactions (Flint and Mott, 2001). These limitations have facilitated the
20 development of novel technologies to simultaneously identify genomic loci that are involved in complex traits. With these technologies, phenotypes like high-temperature tolerance, efficient sporulation and chemical resistance have been genetically unraveled (Steinmetz et al, 2002; Deutschbauer and Davies, 2005; Ehrenreich et al., 2010).

Recently, Swinnen et al. (2012) developed such a strategy, which was successfully employed
25 to identify genetic determinants that are involved in high ethanol tolerance in the yeast *Saccharomyces cerevisiae*. In this strategy, called pooled-segregant whole-genome sequence analysis, it was demonstrated that QTLs underlying a complex trait can be mapped using small populations of segregants. However, the identification of causative mutations in these QTLs remains cumbersome since this method results in a relatively large size of the identified loci,
30 which infers the analysis of a large number of genes. Reducing the size of QTLs can be achieved with inbreeding crosses, as was recently described by Parts et al (2011). However, the use of very large pools makes it an extensive procedure, especially since phenotyping industrially relevant traits often requires elaborate procedures, making the use of large

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numbers of segregants undesirable. Furthermore, although inbreeding crosses can be used to decrease the size QTLs, it remains unknown how it influences the mapping of minor loci.

In order to investigate the effect of inbreeding crosses on QTL mapping of industrially relevant strains, we have applied the pooled-segregant whole-genome sequencing analysis methodology on F1 and F7 segregants of a cross between a yeast strain that is superior for acetic acid tolerance and an industrial strain that is inferior for the same trait. Acetic acid tolerance is an industrially important characteristic as yeast fermentation is severely inhibited by this weak organic acid. As mentioned above, the presence of acetic acid in lignocellulosic hydrolysate strongly affects the fermentative capacity of yeast (Casey et al., 2010; Huang et al., 2011; Narendranath et al, 2001; Taherzadeh et al., 1997; Almeida et al., 2007). Especially the fermentation of pentose sugars suffers from the presence of acetic acid (Casey et al., 2010; Bellissimi et al, 2009; Matsushika and Sawayama, 2012), emphasizing the importance of high acetic acid tolerance to enable efficient conversion of all sugars in lignocellulosic hydrolysate into ethanol. However, multiple attempts to rationally engineer increased acetic acid tolerance in yeast were met with limited success as a high number of genes is involved in the response to acetic acid stress (Abott et al., 2007; Mira et al., 2010 a & b; Li and Yuan, 2010, Hasunuma et al., 2011; Zhang et al., 2011) . Random approaches such as evolutionary engineering has rendered improved strains in terms of acetic acid tolerance (Koppram et al., 2012; Wright et al., 2011), but this method leads to overselection of a single trait and to possible loss of other important properties.

We found for the first time that increased recombination frequency indeed results in the expected smaller loci, but also in unexpected appearance and disappearance of QTLs, compared to QTL mapping without inbreeding crosses. Furthermore, combining individual whole-genome sequencing data of acetic acid tolerant segregants with bioinformatics analysis enabled QTL mapping to single gene level.

Surprisingly we found that a specific allele of *GLO1* is needed and sufficient to confer tolerance to relatively high concentrations of acetic acid. Replacement of the inferior allele by a superior allele results in a significant improvement of the fermentation performance in presence of at least 0.5% acetic acid.

A first aspect of the invention is the use of *GLO1* to modulate the acetic acid tolerance in yeast. Preferably, said use is the use of a specific allele of *GLO1* to increase the acetic acid tolerance, even more preferably said specific allele is encoding SED ID No.2, even more preferably said specific allele consist of SEQ ID No.1.

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In a preferred embodiment, the use according to the invention is the overexpression of the protein, encoded by the specific allele. Such overexpression can be obtained by any method known to the person, skilled in the art. As a non-limiting example, overexpression can be obtained by incorporating more than one copy of the specific allele in a strain, or by placing the coding sequence of the specific allele under control of a strong promoter. In another preferred embodiment, said use according to the invention is the replacement of an inferior allele by the allele according to the invention.

Acetic acid tolerance as used here means that the fermentation performance of the strain in presence of acetic acid is better than that of a control strain with the same genetic background, except for the *GLO1* allele. The concentration of acetic acid in the medium is at least 0.4%, preferably at least 0.5%, more preferably at least 0.6%, even more preferably at least 0.7%, most preferably at least 0.8%. An improved fermentation performance may be measured as a higher ethanol yield, a faster fermentation rate of a shorter lag phase. Preferably said improved fermentation performance is a faster fermentation rate and/or a shorter lag period.

A *GLO1* allele is called a "superior *GLO1* allele" herein if, in a strain with an identical background, except for the *GLO1* allele, the presence of the *GLO1* allele allows improved fermentation performance in the presence of at least 0.4% acetic acid in the medium as compared to a relevant control. Analogously, a *GLO1* allele is termed an "inferior *GLO1* allele" herein if, in a strain with an identical background, except for the *GLO1* allele, the presence of the *GLO1* allele results in worse fermentation performance in the presence of at least 0.4% acetic acid in the medium as compared to a relevant control. The same applies for higher concentrations of acetic acids, e.g. 0.5%, 0.6%, 0.7%, 0.8%.

Preferably, said yeast according to the invention is a xylose fermenting yeast. A xylose fermenting yeast, as used here, can be a yeast that is naturally producing ethanol on the base of xylose, or it can be a yeast that is mutated and/or genetically engineered to ferment xylose and to produce ethanol on the base of xylose. Even more preferably, said yeast is selected from the group consisting of *Saccharomyces* sp., *Pichia* sp., *Candida* sp., *Pachysolen* sp. and *Spathaspora* sp. Most preferably, said yeast is a *Saccharomyces* sp. preferably a *Saccharomyces cerevisiae*.

Another aspect of the invention is a recombinant yeast strain, comprising a recombinant allele encoding SEQ ID No.2. In a preferred embodiment, said recombinant yeast strain comprises a recombinant allele consisting of SEQ ID No. 1.

Still another aspect of the invention is a method to obtain an acetic acid tolerant yeast, by crossing in a superior *GLO1* allele. Crossing in, as used here, can be by classical breeding,

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either by making a heterozygous diploid (comprising an inferior and a superior allele), of by mating and sporulation, selecting the strain comprising the superior allele. In a preferred embodiment, crossing in comprises the replacement of an inferior *GLO1* allele by a superior *GLO1* allele. In a preferred embodiment, said superior *GLO1* allele is encoding SEQ ID No.2.

5 Preferably, said superior *GLO1* allele is consisting of SEQ ID No.1.

Still another aspect of the invention is a method for selecting acetic acid tolerant yeast, comprising the identification of the presence of a superior *GLO1* allele. Said identification can be done by any method known to the person skilled in the art. Preferably, said method comprises the sequencing of the *GLO1* allele. Preferably, said superior *GLO1* allele is encoding SEQ ID No.2. Even more preferably, said superior *GLO1* allele is consisting of SEQ ID No.1.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Small scale fermentation of RHA strains for *GLO1*. The sugar conversion is expressed as % weight loss.

15 **Figure 2:** Replacement of *GLO1* in ER18 by *GLO1* from 16D (A) replacement of *GLO1* in ER18 by a kanamycin marker; (B) removal of the kanamycin cassette (C) Tagging of *GLO1* in 16D by a kanamycin cassette and isolation of the *GLO1* kanamycin fragment by PCR (D) Transformation of the tagged fragment in ER18 and removal of the kanamycin cassette.

20 **Figure 3:** Fermentation performance of the parental ER18 strain, compared with the ER18 variant in which the original inferior *GLO1* allele has been replaced by a superior 16D allele

EXAMPLES

Material and methods to the examples

Strains used in the study

25 Ethanol red is a diploid industrial strain, and was obtained from Fermentis. The strain was sporulated, and the haploid segregants ER18 and 16D have been isolated on the base of the difference in their acetic acid resistance (see Table I)

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Table I

Stains used for RHA analysis GLO1		
Name	Genotype	Stocknumber
ER18	Inferior parent	JT 24050
16D	Superior parent	JT 24211
ER18 x 16D	Hybrid of ER18 and 16D	JT 24198
BY4741 <i>glo1Δ::KanMX4</i>	Isogenic to BY4741; except <i>glo1 Δ::KANMX</i>	JT_a.390
16D <i>glo1Δ::KanMX4 colony1</i>	Isogenic to 16D; except <i>glo1 Δ::KANMX</i>	PV_T1
16D <i>glo1Δ::KanMX4 colony2</i>	Isogenic to 16D; except <i>glo1 Δ::KANMX</i>	PV_T2
ER18 <i>glo1Δ::KanMX4 colony1</i>	Isogenic to ER18; except <i>glo1 Δ::KANMX</i>	PV_T3
ER18 <i>glo1Δ::KanMX4 colony2</i>	Isogenic to ER18; except <i>glo1 Δ::KANMX</i>	PV_T4
ER18 x 16D <i>glo1Δ::KanMX4 colony1</i>	Hybrid of ER18 and 16D <i>glo1Δ::KanMX4 colony1</i>	PV_T5
ER18 x 16D <i>glo1Δ::KanMX4 colony2</i>	Hybrid of ER18 and 16D <i>glo1Δ::KanMX4 colony2</i>	PV_T6
ER18 <i>glo1Δ::KanMX4</i> x 16D colony1	Hybrid of 16D and ER <i>glo1Δ::KanMX4 colony1</i>	PV_T7
ER18 <i>glo1Δ::KanMX4</i> x 16D colony2	Hybrid of 16D and ER <i>glo1Δ::KanMX4 colony2</i>	PV_T8

Construction of RHA strains

- 5 The reciprocal deletions were engineered in the haploid strains, after which the proper haploids were crossed to obtain the diploid hybrids. The haploid deletion strains were created by gene targeting in the parental strains 16D and ER18. Deletion cassettes were PCR amplified from genomic DNA of strain BY4741 *glo1Δ::KANMX4* (JT_a.390), obtained from the deletion collection (Winzeler et al., 1999), and primers B-2344 and B-2345. After
- 10 transformation with the lithium acetate method (Gietz et al., 1995), transformants were selected on YPD plates containing geneticin (200mg/l). Deletion of GLO1 was confirmed by PCR with primercouple A-3863/B-2612. Of each transformed strain, two transformants were selected and subsequently crossed with the corresponding parental strain to construct the hybrid diploid strains. Mating type of the diploids was confirmed by diagnostic PCR for the
- 15 MAT locus (Huxley et al., 1990).

Assessment of acetic acid tolerance

Acetic acid tolerance in media containing acetic acid and glucose was evaluated by determination of the fermentation performance of yeast strains in small scale, near anaerobic batch fermentations. Yeast strains were pre-cultured in YPD medium (30°C, static incubation

20 and 60 hour). After collection (1700 g, 2 minutes) and washing of the cells with Milli-Q water, cylindrical glass tubes containing 100 ml of YP medium supplemented with 4% w/v D-glucose and a range of acetic acid, adjusted to pH=4 with HCL or KOH, were inoculated at an OD600 of 0.3. The culture was agitated continuously at 120 rpm using a magnetic rod. The

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fermentations were performed at 30°C. The course of the fermentation was monitored by weighing the fermentation tubes at regular intervals.

Example 1: Screening for superior acetic acid tolerance

Ethanol Red is a diploid yeast strain that is being used for bio-ethanol production at high
5 temperatures, showing ethanol yields of up to 18 %. However, the fermentation performance of
this industrial yeast strain is severely affected by acetic acid, a weak organic acid present in
high quantities in lignocellulosic hydrolysates. Haploid segregants were isolated from this yeast
strain and scored on acetic acid tolerance by fermentation in YPD medium supplemented with
various concentrations of acetic acid. It was observed that the maximum tolerance of Ethanol
10 Red towards acetic acid was 0.6 % (v/v) in YPD medium at a pH of 4.0. However, the lag
phase was significantly prolonged by adding acetic acid to the growth medium, with a lag
phase of approximately 30 hours at concentrations of 0.5 % and 0.6 %. The haploid Ethanol
Red segregant #18 (named ER18) showed similar tolerance to acetic acid and was therefore
selected for further experiments.

15 In order to obtain a yeast strain with high acetic acid tolerance, the in-house yeast collection
and the yeast collection from the Fungal Biodiversity Centre (CBS-KNAW, Utrecht, The
Netherlands) were screened under acetic acid conditions. More than 1000 yeast strains were
assessed, from which strain JT 22689 showed the best performance under fermentative
conditions at high acetic acid concentrations, being able to ferment glucose in the presence of
20 0.9 % acetic acid without a lag phase (not shown). Also from this strain a haploid segregant,
named 16D, could be isolated that showed a similar phenotype in terms of acetic acid
tolerance.

Example 2: QTL mapping with pooled F1 segregants

Mapping the genetic determinants that are responsible for the high acetic acid tolerance of
25 16D was initiated by crossing the haploid segregants ER18 and 16D. The resulting hybrid
strain was subsequently sporulated to obtain segregants that contain a mixture of the parental
genomes. Obtained segregants were subsequently screened for high acetic acid tolerance,
resulting in the identification of 27 (out of 288) segregants that were able to ferment glucose in
the presence of 0.9 % acetic acid, which is comparable with the tolerance observed for the
30 superior parent strain. These 27 segregants were therefore selected for pooled-segregant
whole-genome sequencing analysis. Genomic DNA isolated from the two parent strains, a pool
of the 27 selected segregants and a control pool of 27 randomly selected segregants was sent
for custom sequencing analysis using the Illumina HiSeq2000 technology (BGI, Hong Kong,
China). The sequence reads from parent strains ER18 and 16D were aligned with the

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- reference sequence from strain S288C. A total number of 23,150 SNPs between ER18 and 16D could be identified, which were subsequently filtered according to the method described by Duitama et al. (2012). The SNP variant frequencies were calculated by dividing the number of the alternative variant by the total number of aligned reads. The calculated variant frequencies were subsequently plotted against the respective chromosomal positions. The underlying structure in the SNP variant frequencies scatterplot of a given chromosome was identified by fitting smoothing splines in the generalized linear mixed model framework, as described by Claesen et al. (2013). Variant frequencies that significantly deviate from 50% (random segregation) are indicative of genetic linkage to the phenotype.
- The results from the QTL mapping show two loci on the genome with a strong linkage to the superior segregant 16D: QTL1 on chromosome XIII and a second QTL on chromosome XVI. The statistical significance of QTL1 was confirmed using the Hidden Markov Model described previously, stretching from position 181019 – 294166. Both QTLs were further investigated by scoring selected SNPs in the 27 individual segregants in order to precisely determine the SNP variant frequencies and the statistical significance of the genetic linkage. Using a binomial test previously described (Swinnen et al., 2012; Claessen et al., 2013), both loci were found to be statistically significant. Furthermore, the size of both QTLs could be decreased to regions stretching from roughly 224000 – 277000 for QTL1 on chromosome XIII, and 568000 – 615000 for QTL2 on chromosome XVI.
- GLO1* was confirmed as causative gene for acetic acid tolerance by RHA

Example 3: Fermentation assay of RHA strains

Figure 1 shows the fermentation profiles of the RHA strains for *GLO1*. Every point represents the average of two biological repeats. The error bars indicate the standard error of the mean.

- The strains with at least one allele originating from the 16D strain show superior fermentation performance in presence of acetic acid.

Example 4: Replacement of the *GLO1* allele from strain ER18 by the allele from strain 16D

- In order to upgrade the *GLO1* allele of ER18, a fragment comprising the ORF, 631bp upstream and 44bp downstream of the ORF of *GLO1* was replaced by its 16D counterpart. The method to replace the allele comprises three steps:

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1. Deletion of the region containing the ORF of *GLO1*, 631 bp upstream and 44 bp downstream in ER18. Primers B-2610 and B-2609 are used to amplify the deletion cassette from plasmid pJET1,2-AttB-KANMX-AttP.

Both primers contain a 19bp region, binding to pJET1,2-B-KANMX-P and 50bp tails that are homologous to the nucleotides flanking the region that needs to be deleted. In the schematic representation of Figure 2A, these homologous regions are shown as light grey boxes. After transformation, colonies will be selected on YPD plates containing geneticin (200mg/l). Hereafter, colonies were confirmed by PCR with primer couple A-3863/B-2612.

Hereafter, strain ER18 *glo1Δ::KANMX4* was transformed with plasmid pBEVY-nat-Phic31integrase. After selection on YPD plates containing nourseothricin (100mg/l), the kanamycin marker was removed due to the action of the phage derived phiC31 integrase, leaving an AttL sequence at the recombination site (Figure 2B).

After confirming of the loss of the KANMX marker by checking the lack of growth on YPD geneticin plates, the strain was cured of the plasmid by growing several rounds in liquid YPD medium.

2. Next, 16D was tagged by a kanamycin marker, 631 bp upstream of *GLO1*. As in step 1, primers were used that contain a 19bp region binding to pJET1,2-B-KANMX-P and 50bp tails that are homologous to the regions flanking the location where the marker needs to be inserted. The primers used for amplification of the cassette from pJET1,2-B-KANMX-P are B-2610 and B-2827.

After transformation of this fragment, selection on YPD plates containing geneticin and confirmation of the colonies by PCR with primer couple A-3863/B-2612, genomic DNA of this strain was used as a template for amplification of the tagged *GLO1_16D* allele. Primers B-2965 and B-2611 were used for amplification of the tagged *GLO1_16D* allele. (Figure 2C)

3. Finally, the PCR product of the tagged *GLO1_16D* allele, containing the *GLO1* allele of 16D linked to a KANMX cassette, was transformed in ER18 *glo1Δ::AttL*, the strain obtained after step 1. After transformation of this fragment, selection on YPD plates containing geneticin and confirmation of the colonies by PCR with primer couple A-3863/B-2612, the KANMX cassette is removed by the action of the phiC31 integrase (described previously). (Figure 2D)

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Example 5: The *GLO1* allele from strain 16D is needed and sufficient to confer acetic acid tolerance

The fermentation profiles of the ER18 parental strain, and the ER18 strain in which the original *GLO1* allele has been replaced by an 16D *GLO1* allele are shown in Figure 3. Every point represents the average of two biological repeats. The error bars indicate the standard error of the mean. ER18 is the original inferior parent. ER18 *glo1Δ::GLO1_16D* is the ER18 strain in which the *GLO1* gene comprising the ORF, 631 bp upstream and 44 bp downstream of the ORF of *GLO1* were replaced by its 16D counterpart.

10 **Table II. Presence of non-synonymous mutations and the corresponding codons and encoded amino acids in *GLO1* from different *S. cerevisiae* strains for which the whole genome sequence is available.**

<i>GLO1</i>				
	nt (+106-108)	aa (36)	nt (+964-966)	aa (322)
ER18	ACC	T	CAT	H
16D	GCT	A	TAT	Y
S288C	ACC	T	CAT	H
AWRI1631	GCT	A	TAT	Y
AWRI796	GCT	A	TAT	Y
BY4741	ACC	T	CAT	H
BY4742	ACC	T	CAT	H
CBS7960	GCT	A	CAT	H
CEN.PK113	ACC	T	CAT	H
CLIB215	GCT	A	TAT	Y
EC1118	GCT	A	TAT	Y
EC9-8	GCT	A	TAT	Y
FL100	ACC	T	CAT	H

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GLO1				
	nt (+106-108)	aa (36)	nt (+964-966)	aa (322)
FostersB	GCT	A	CAT	H
FostersO	GCT	A	CAT	H
JAY291	GCT	A	CAT	H
Kyokai7	ACC	T	CAT	H
LalvinQA23	GCT	A	-----	-
PW5	ACT	T	CAT	H
RM11-1a	GCT	A	TAT	Y
Sigma1278b	ACC	T	CAT	H
T7	ACT	T	CAT	H
UC5	ACC	T	CAT	H
VL3	-----	-	TAT	Y
Vin13	GCT	A	TAT	Y
W303	ACC	T	CAT	H
YJM269	ACC	T	CAT	H
YJM789	ACT	T	CAT	H
ZTW1	ACC	T	TAT	Y

GLO1 of strain LalvinQA23 has an early stop codon resulting in a truncated ORF that lacks amongst others nt 964-965 and codes for a truncated protein that lacks amongst others aa 322.

5

GLO1 of strain VL3 lacks nucleotides 1 – 558 of the ORF and starts with the ATG at position 559-561 resulting in a shortened protein. Hence, it lacks nt 106 – 108 and aa 36, but not nt 964 – 966 and aa 322.

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Table III: *GLO1* Promoter mutations: comparison of strains

	-782	-775	-645b	-645a	-562	-559	-531	-460	-431	-385b	-385a	-384	-273	-230	-219	-135	-77	-64	-48
ER18	A	A	-	-	T	G	C	C	A	A	T	T	C	A	C	C	-	G	T
16D	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
S288C	A	G	-	-	C	G	T	T	G	-	-	C	C	A	C	C	A	A	T
AWRI1631	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
AWRI796	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	T
CBS7960	G	G	C	A	C	A	C	T	G	A	T	T	T	A	C	G	A	G	-
CEN.PK113	A	G	-	-	C	G	T	T	G	-	-	C	C	A	C	C	A	A	T
CLIB215	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
EC1118	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
EC9-8	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
FL100	A	G	-	-	C	A	C	T	G	A	T	T	T	T	T	G	A	A	T
FostersB	G	G	C	A	C	A	C	T	G	A	T	T	T	W*	Y**	G	A	G	-
FostersO	G	G	C	A	C	A	C	T	G	A	T	T	T	W*	Y**	G	A	G	-
JAY291	G	G	C	A	C	A	C	T	G	A	T	T	T	A	C	G	A	G	-
Kyokai7	A	A	-	-	T	G	C	C	G	A	T	T	C	A	C	C	A	G	T
LalvinQA23	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
PW5	A	G	-	-	C	A	C	T	G	A	T	T	T	A	C	G	A	G	-
RM11-1a	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
Sigma1278b	A	G	-	-	C	G	T	T	G	-	-	C	C	A	C	C	A	A	T
T7	A	G	-	-	C	A	C	T	G	A	T	T	T	A	C	G	A	G	-
UC5	A	A	-	-	T	G	C	C	G	A	T	T	C	A	C	C	A	G	T
VL3	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
Vin13	G	G	C	A	C	A	C	T	G	A	T	T	T	T	T	G	A	G	-
W303	A	G	-	-	C	G	T	T	G	-	-	C	C	A	C	C	A	A	T
YJM269	A	G	-	-	C	G	T	T	G	-	-	C	C	A	C	C	A	G	T
YJM789	A	G	-	-	C	A	C	T	G	A	T	T	T	A	C	G	A	G	-
ZTW1	A	A	-	-	T	G	C	C	G	A	T	T	C	A	C	C	A	G	T

* W : A, T or U

** Y: C, T or U

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CLAIMS

1. The use of *GLO1* for obtaining acetic acid tolerance in yeast.
2. The use of *GLO1* according to claim 1, wherein said *GLO1* is an allele encoding SEQ
5 ID NO: 2.
3. The use of *GLO1* according to claim 2, wherein said *GLO1* consists of SEQ ID NO: 1.
4. The use according to any of the preceding claims, wherein said use is replacement of
an inferior allele by a superior allele.
5. The use according to any of the preceding claims, wherein said use is an
10 overexpression.
6. The use according to any of the preceding claims, wherein said yeast is a
Saccharomyces species.
7. A recombinant yeast strain, comprising a recombinant allele encoding SEQ ID NO: 2.
8. The yeast strain according to claim 7, wherein said yeast is a *Saccharomyces* species.
9. A method to obtain an acetic acid tolerant yeast, comprising the replacement of an
15 inferior *GLO1* allele by a superior *GLO1* allele.
10. A method for selecting acetic acid tolerant yeast, comprising the identification of the
presence of a superior *GLO1* allele.

Figure 1

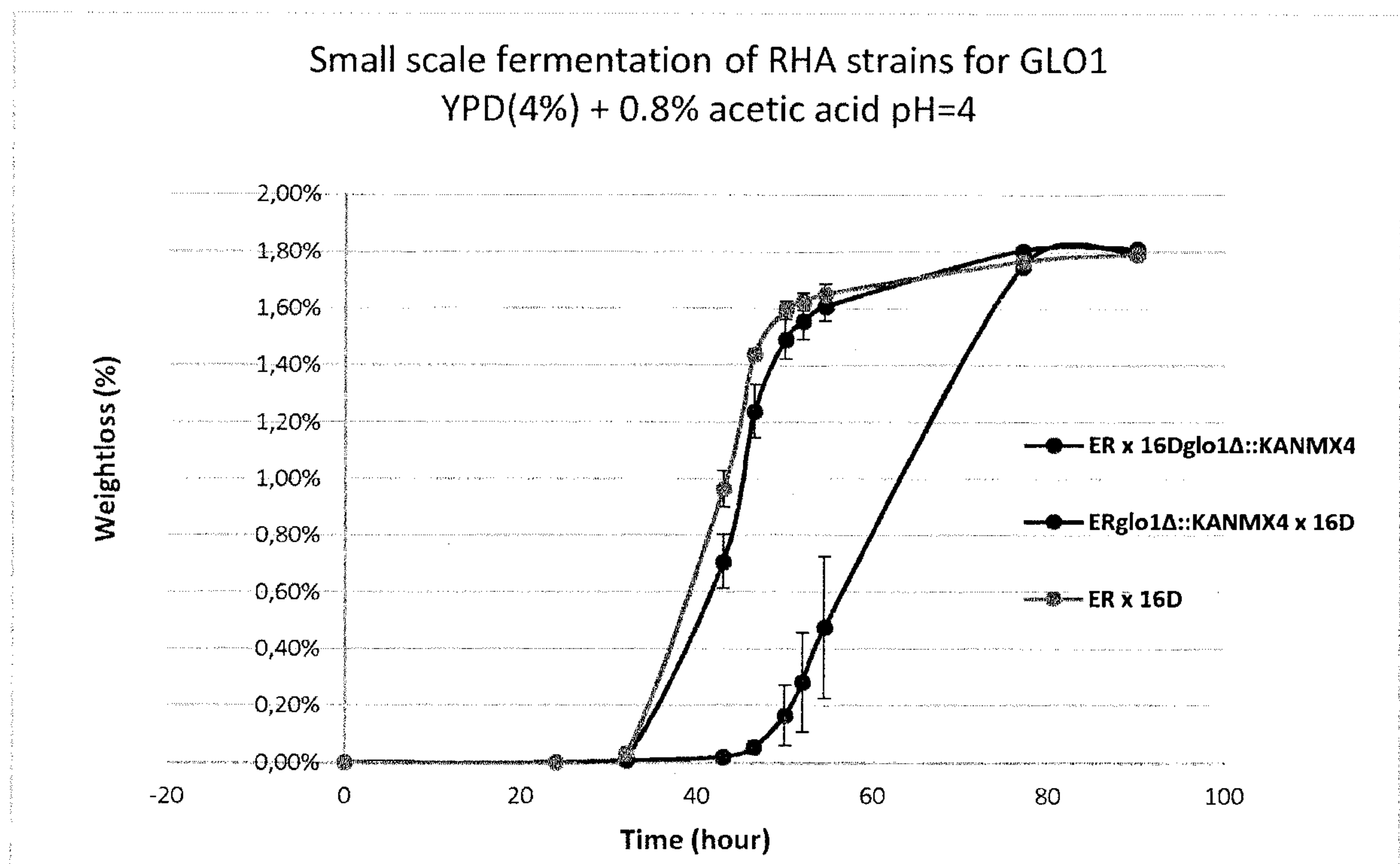


Figure 2

A

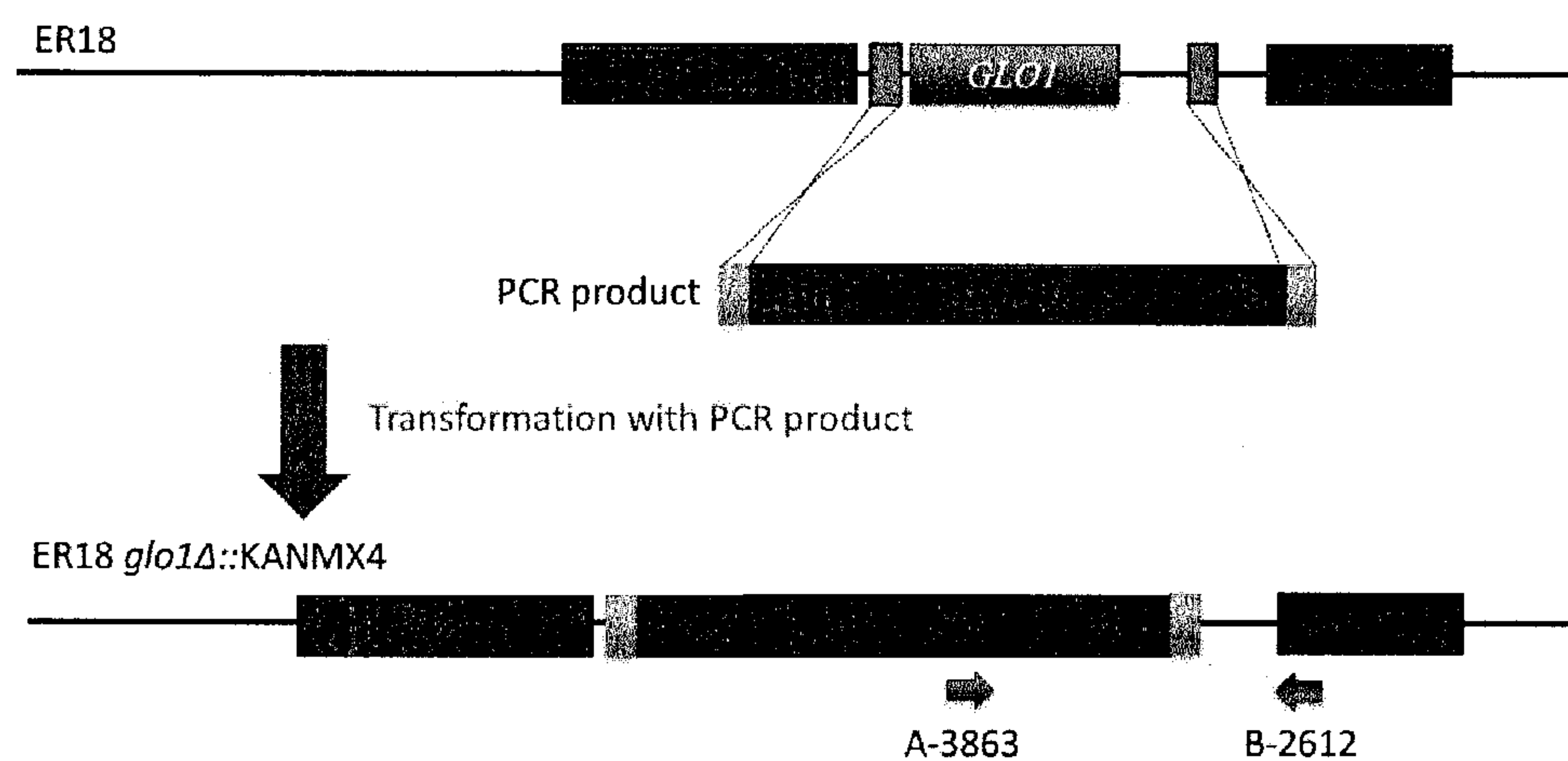
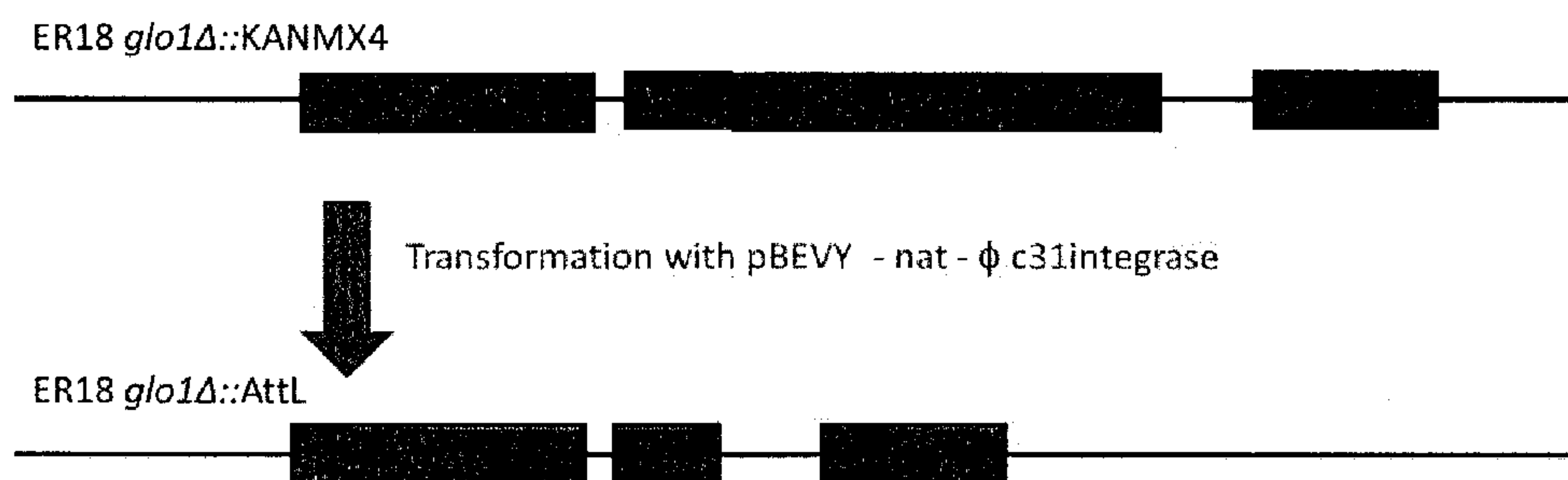
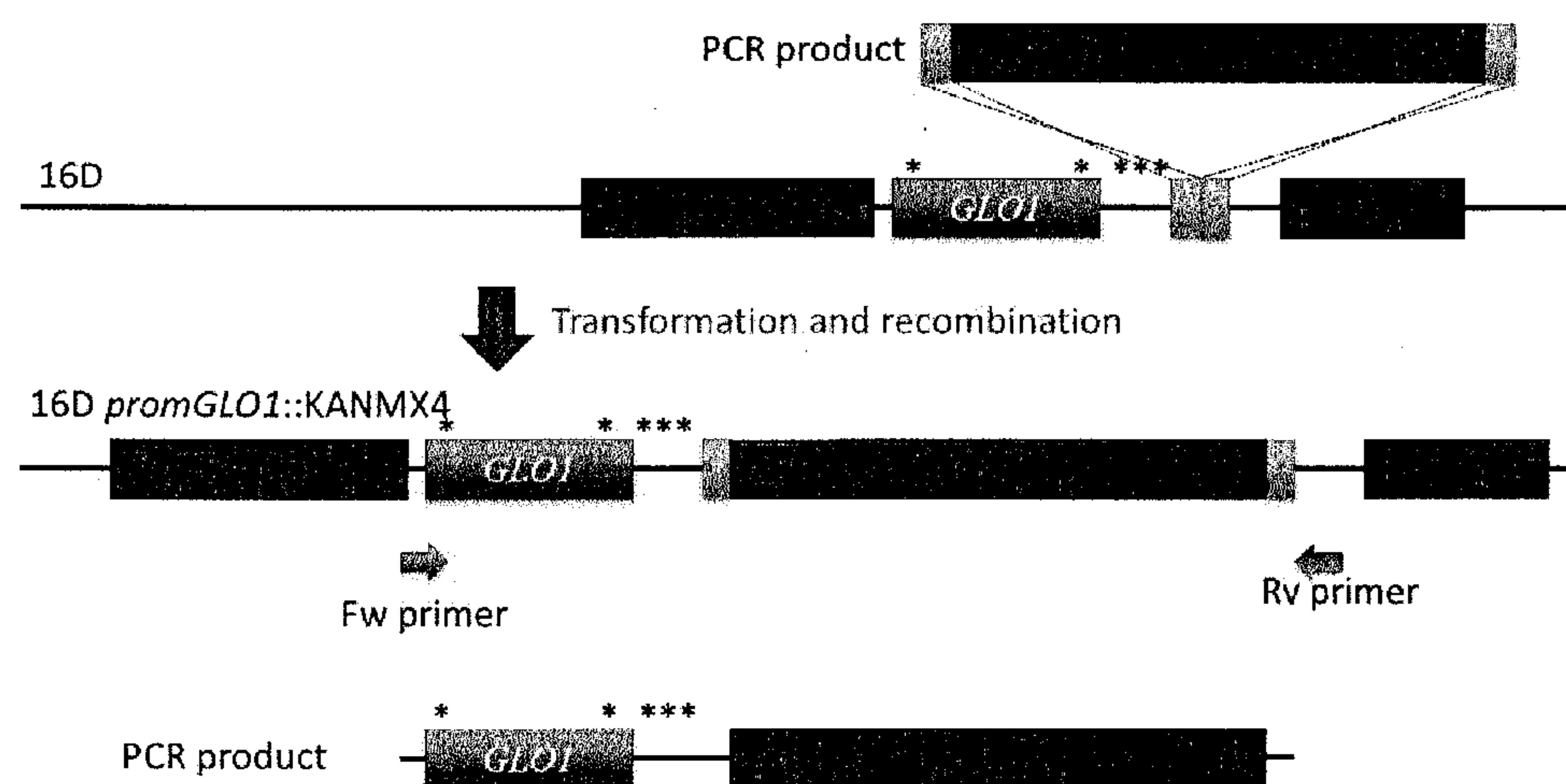


Figure 2 continued

B



C



D

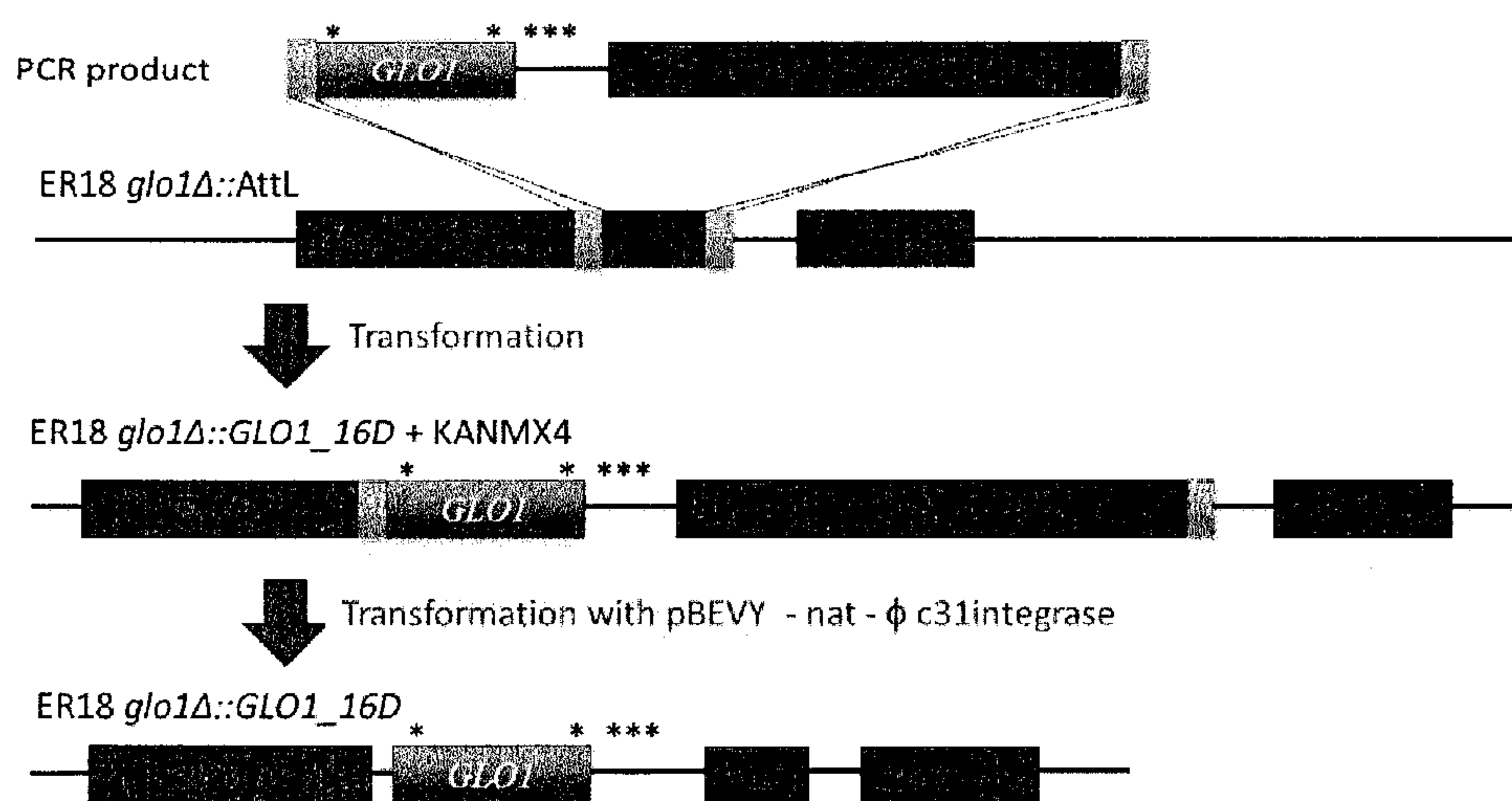


Figure 3

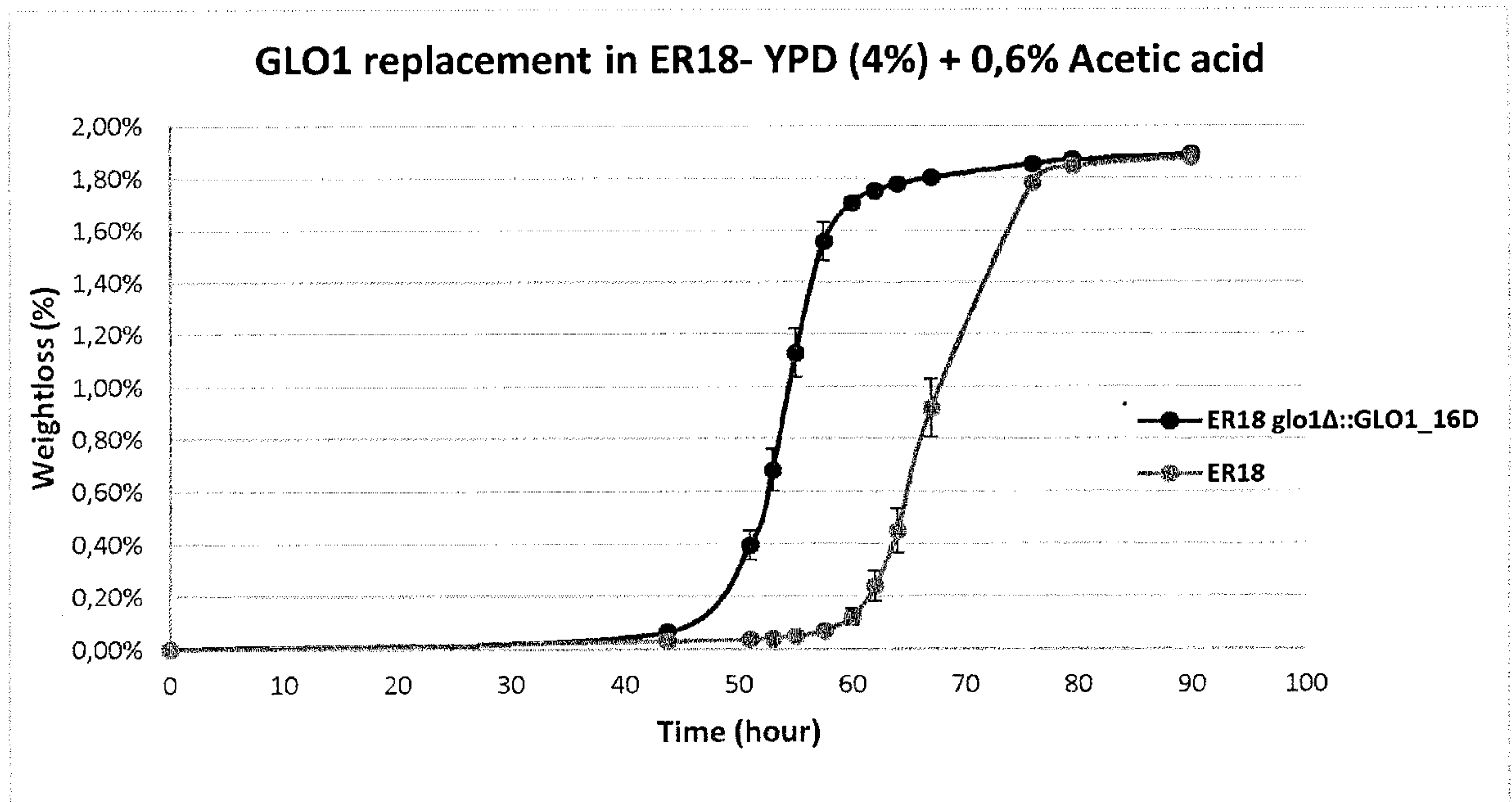


Figure 3

