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Unravelling the genetic basis of ethanol tolerance and maximal ethanol accumulation capacity in yeast for very-high gravity bioethanol production

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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Science: Biochemistry and Biotechnology

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SUMMARY

Concerns about global warming and climate change due to greenhouse gas emissions and the growing energy demand for transportation, heating and industrial processes has encouraged the search for sustainable renewable energy sources. Today, bioethanol is the most common biofuel mainly derived from starch or sugar cane and shows great potential as a building block for bio-based chemicals such as bioplastics. *Saccharomyces cerevisiae* is currently the preferred micro-organism for bioethanol production due to its highly efficient ethanol productivity and high tolerance to various fermentation-associated stresses such as high ethanol concentrations. Although much research has been done to understand the genetic basis of high ethanol tolerance in yeast, most of the research was focused on low and moderate ethanol tolerance in laboratory yeast strains. Previous research in our laboratory has been initiated to study high ethanol tolerance and maximal ethanol accumulation capacity in industrial yeast strains by pooled-segregant whole-genome sequence analysis in order to find genetic determinants that contribute to both of these important industrial traits.

In the first part of this thesis, we have further explored the genetic basis of high ethanol tolerance in a former Brazilian bioethanol production strain VR1 which was originally initiated by the research of Dr. S. Swinnen. Our results indicate that very high ethanol concentrations generally compromise the uptake of nutrients required to fulfil auxotrophic requirements and that therefore auxotrophic mutations lead to lower ethanol tolerance. We have downscaled the last major QTL on chromosome X and identified *VPS70* as a new causative allele that contributes to high ethanol tolerance. Additionally, we have confirmed that *APJ1* has a negative effect on ethanol tolerance and that a lower expression or deletion of the *APJ1* allele improves ethanol tolerance. Introduction of all superior alleles in the inferior background has succeeded in improving ethanol tolerance of the inferior parent strain (BY) to an even higher level of that in the superior parent (VR1-5B).

In the second part of this thesis, we have further described the genetic dissection of maximal ethanol accumulation capacity of a sake strain CBS1585. We have identified two more causative genes *PTK2* and *SHE4*, supporting superior maximal ethanol accumulation capacity, which expands our understanding of the genetic determinants involved in general ethanol

tolerance as well as in maximal ethanol accumulation capacity in very high gravity fermentations. Unfortunately, we could not improve maximal ethanol accumulation in the strain V1116 wine strain by overexpression of the most promising target genes identified during pooled-segregant gene expression analysis upon stable integration in the genome. However, further engineering in order to obtain higher expression of the integrated constructs has a clear potential to provide positive results for improving maximal ethanol accumulation capacity in a stable manner.

Finally, we have introduced a specific combination of causative alleles in the ER18 strain which could clearly improve this strain for ethanol tolerance of cell proliferation and maximal ethanol accumulation capacity. However, it still remains to be investigated to what extent these causative genes are universal determinants so that they can be used to improve other industrial yeast strains for bioethanol production. Altogether, this work has provided new insights into the genetic basis of high ethanol tolerance and maximal ethanol accumulation capacity of industrial yeast strains.

SAMENVATTING

De verontrusting over de wereldwijde opwarming van de aarde en de klimaatsverandering als gevolg van de uitstoot van broeikasgassen en de bovendien steeds maar groeiende vraag naar energie voor transport, verwarming en industriële processen heeft de zoektocht naar duurzame energiebronnen aangemoedigd. Vandaag is bio-ethanol de belangrijkste biobrandstof afkomstig van zetmeel houdende gewassen of suikerriet en toont hierbij groot potentieel als bouwsteen voor bio-chemicaliën zoals bio-plastics. Momenteel is *Saccharomyces cerevisiae* bij voorkeur het micro-organisme voor bio-ethanol productie vanwege zijn zeer efficiënte ethanol productiviteit en hoge tolerantie aan verschillende stressfactoren tijdens het fermentatieproces zoals bv. hoge ethanol concentraties. Hoewel in het verleden veel onderzoek gedaan is om de genetische achtergrond van hoge ethanol tolerantie in gist te begrijpen, was het merendeel van het onderzoek gericht op lage en matige ethanol tolerantie in laboratorium giststammen. In ons laboratorium is voorafgaand onderzoek gestart om de hoge ethanol tolerantie en maximale ethanol accumulatie capaciteit in industriële giststammen te bestuderen door *pooled-segregant whole-genome sequence analysis* toe te passen zodat we genetische determinanten kunnen identificeren die bijdragen aan deze belangrijke industriële eigenschappen van de giststam.

In het eerste deel van deze thesis, hebben we de genetische basis van hoge ethanol tolerantie in een voormalige Braziliaanse bio-ethanol productiestam VR1 verder onderzocht. Dit onderzoek werd initieel opgestart door Dr. S. Swinnen. Onze resultaten tonen aan dat zeer hoge ethanol concentraties de opname van voedingsstoffen belemmert, die nodig is om aan auxotrofe noden te voldoen, wat leidt tot een verlaagde ethanol tolerantie. Verder hebben we *VPS70* geïdentificeerd als een nieuw causatief gen in chromosoom X dat bijdraagt tot een hoge ethanol tolerantie. Daarnaast hebben we bevestigd dat *APJ1* een negatief effect heeft op de ethanol tolerantie en dat een verlaagde expressie of deletie van *APJ1* de ethanol tolerantie verbetert. Introductie van al de superieure allelen in de inferieure ouderstam (BY) heeft geleid tot een verbetering van de ethanol tolerantie die zelfs hoger werd dan die van de superieure ouderstam (VR1-5B).

In het tweede deel van de thesis beschrijven we de genetische analyse van maximale ethanol accumulatie capaciteit in de CBS1585 sakéstam. Twee nieuwe causatieve allelen, *PTK2* en *SHE4*, werden geïdentificeerd die bijdragen tot een maximale ethanol accumulatie capaciteit. Daardoor wordt onze kennis verhoogd over de genetische determinanten betrokken bij zowel ethanol tolerantie als maximale ethanol accumulatie capaciteit in zeer hoge graviteitsfermentaties. Helaas zijn we er niet in geslaagd om de maximale ethanol accumulatie capaciteit in de V1116 wijnstam te verbeteren door overexpressie van de veelbelovende target genen die geïdentificeerd werden tijdens de *pooled-segregant* genexpressie analyse. Echter, optimalisatie door een stabiele verhoogde expressie van de target genen in het genoom heeft duidelijk potentieel om positieve resultaten te bekomen voor verbetering van de maximale ethanol accumulatie capaciteit

Tot slot werd een specifieke combinatie van causatieve allelen in de ER18-stam geïntroduceerd die de ethanol tolerantie en maximale ethanol accumulatie capaciteit hebben verbeterd. Verder onderzoek zal moeten uitmaken in welke mate deze causatieve genen universeel gebruikt kunnen worden voor de verbetering van deze eigenschappen in andere giststammen. In het algemeen heeft dit werk geleidt tot nieuwe inzichten in de genetische basis van hoge ethanol tolerantie en maximale ethanol accumulatie capaciteit in industriële giststammen.

LIST OF ABBREVIATIONS

1G	First generation
2G	Second generation
3G	Third generation
ATP	Adenosine triphosphate
Bio-DME	Bio-dimethyl ether
Bio-SNG	Bio-syngas
(b)RHA	(bulk) Reciprocal Hemizygosity Analysis
cAMP	cyclic adenosine monophosphate
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DDGS	Distillers dried grains and solubles
ED pathway	Entner-Doudoroff pathway
FT-diesel	Fisher-Tropsch diesel
GHG	Greenhouse gases
GO	Gene ontology
gTME	Global transcription machinery engineering
HMM	Hidden Markov Model
HSE	Heat shock element
HSP	Heat shock protein
IEA	International Energy Agency
mRNA	messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PKA	Protein kinase A
QTL	Quantitative trait locus/loci
ROS	Reactive oxygen species
S. cerevisiae	Saccharomyces cerevisiae
SGKO	Single gene knockout
SHF	Separate hydrolysis and fermentation
SNP	Single nucleotide polymorphism
SSF	Simultaneous saccharification and fermentation
STRE	Stress-responsive element
TCA-cycle	Tricarbocylic acid cycle
TF	Transcription factor
TOR	Target of rapamycin
UDP	Uridine diphosphate
UFA	Unsaturated fatty acids
VHG	Very high gravity

TABLE OF CONTENTS

Acknowledgments I

Summary III

Samenvatting V

List of abbreviations VII

Table of contents VIII

Introduction 1

CHAPTER I Literature overview 3

1 Biofuels 5

1.1 Classification of biofuels 6

1.1.1 First generation biofuels 7

1.1.2 Second generation biofuels 8

1.1.3 Third generation biofuels 9

1.2 Bioethanol as a renewable feedstock for bio-based chemicals 10

1.3 Bioethanol production process 11

1.3.1 First generation bioethanol production process 11

1.3.2 Second generation bioethanol production process 11

1.3.3 Very high gravity fermentation 13

1.3.4 Micro-organisms for bioethanol production 14

2 The cellular impact of ethanol stress on *S. cerevisiae* 18

2.1 Ethanol stress and damage to cellular membranes 18

2.1.1 Plasma membrane composition and ethanol damage 18

2.1.2 The role of amino acids 21

2.1.3 Intracellular pH and H^+-ATPases 22

2.1.4 Stabilisation and repair of cell components during ethanol stress 24

2.2 The ethanol stress response 27

2.2.1 Transcriptional response to ethanol stress 27

2.2.2 Functional genomic screens and ethanol stress 29

2.2.3 Signal transduction pathways involved in ethanol stress 32

2.3 Strain improvement for ethanol tolerance and/or ethanol production 34

RESULTS

CHAPTER II 39

1. Introduction 41

2. Results 43

2.1. Auxotrophic mutations reduce tolerance to high ethanol concentrations of *Saccharomyces cerevisiae* 43

2.1.1. Growth specificity of auxotrophic mutations to high ethanol concentrations and other stresses 43

2.1.2. Effect of high ethanol concentrations on nutrient uptake in *S. cerevisiae* 46
2.2. Identification of the causative gene for high ethanol tolerance in QTL2 on chromosome X 50
2.2.1. Fine-mapping of QTL2 and sequence analysis 50
2.2.2. Functional analysis of candidate genes reveals one causative gene for QTL2 52
2.3. Further research on the superior allele *APJ1* for high ethanol tolerance 55
2.3.1. Evaluation of the possible improvement of ethanol tolerance in industrial yeast strains by *APJ1* deletion. 57
2.4. Introduction of the different superior alleles in the inferior parent background 60

3. Discussion 61

4. Own publications on these subjects 66

CHAPTER III 67

1 Introduction 69

2 Results 71
2.1 Genetic dissection of QTL9 on chromosome X linked to maximal ethanol production capacity 71
2.1.1 Fine-mapping QTL9 by bulk reciprocal hemizygosity analysis (bulk RHA) 71
2.2 Genetic dissection of QTL17 on chromosome XV linked to maximal ethanol accumulation capacity and high ethanol tolerance of cell growth 75
2.2.1 Fine-mapping QTL17 by bulk reciprocal hemizygosity analysis (bulk RHA) 75
2.3 Applicability of overexpression of target genes to improve maximal ethanol accumulation capacity in an industrial yeast strain 83

3 Discussion 87

CHAPTER IV 91

1 Introduction 93

2 Results 95
2.1 Characterisation of industrial yeast strains for maximal ethanol accumulation and ethanol tolerance. 95
2.2 Engineering ER18 for higher ethanol tolerance and maximal ethanol accumulation capacity using CRISPR-Cas9 technology 98

3 Discussion 109

Concluding remarks and perspectives 115

Materials and methods 127

Appendix 145

References 151

Introduction

Worldwide, bioethanol is the most used biofuel and serves as a sustainable renewable energy replacement for fossil fuels due to the inevitable exhaustion of crude oil reserves and the concerns about environmental pollution. *Saccharomyces cerevisiae* is the main organism used in bioethanol production due to its high ethanol tolerance, high ethanol productivity, robustness and ability to ferment a wide range of sugars compared to other microbes. However, yeast still encounters strong challenges in very high gravity fermentation which inhibit ethanol production such as the high ethanol level accumulated, high temperature, high osmotic pressure, … Using yeast strains with improved ethanol tolerance could enhance the efficiency of fermentation due to a higher rate and titer of the ethanol production and therefore lead to reduced production costs because of lower liquid volumes in the plant and to reduced bacterial contamination.

Ethanol tolerance and maximal ethanol accumulation capacity are polygenic traits that are controlled by multiple genes. The genomic era has provided a detailed description of the transcriptional response to high ethanol levels and has led to the identification of genes required for ethanol tolerance in laboratory strains. However, it is important to study the polygenic basis of these traits in natural and industrial *S. cerevisiae* strains in order to identify alleles that can be used to further enhance ethanol tolerance. Previous research in our lab has been initiated to understand the genetic basis of both traits. Swinnen and co-workers (2012) applied pooled-segregant whole genome sequence analysis for QTL mapping of high ethanol tolerance in a former Brazilian bioethanol production strain VR1 in order to identify causative mutations underlying high ethanol tolerance. They identified three major QTLs located on chromosome V (QTL1), X (QTL2) and XIV (QTL3) and additional minor QTLs. They succeeded in identifying the causative genes of the two loci with the strongest linkage using reciprocal hemizygosity analysis. The *URA3* gene of the superior parent was identified as the causative allele in QTL1. While in QTL3, three closely located alleles ($MKT1_{VR1\text{-}5B}$, $SWS2_{BY4741}$ and $APJ1_{VR1\text{-}5B}$) were identified as the causative alleles for high ethanol tolerance. The causative genes in the remaining QTLs, however, remained unknown. Pais and co-workers (2013) have applied pooled-segregant whole genome sequence analysis to compare the genetic basis of maximal ethanol accumulation capacity and high ethanol tolerance in the

sake yeast strain, CBS1585. They showed that both traits have a partially different genetic basis and found eleven QTLs that were linked to maximal ethanol accumulation capacity (eight QTLs linked to the superior parent CBS1585 and three QTLs to the inferior parent BY4742). This study identified three genes required for maximal ethanol accumulation capacity, being the superior alleles of the superior parent CBS1585, located in two QTLs: *ADE1* (QTL2, chr. I), *KIN3* (QTL2, chr. I) and *URA3* (QTL3, chr. V).

The goal of the present work is to further reveal the genetic basis of high ethanol tolerance and maximal ethanol accumulation capacity in *Saccharomyces cerevisiae,* which has been initiated by this previous research in our laboratory, in order to identify new causative alleles that contribute to high ethanol tolerance/production in very high gravity fermentations with *S. cerevisiae*. In chapter I, we present a brief overview of the current bioethanol production technologies followed by the relevant literature on the cellular impact of ethanol stress in *S. cerevisiae.* In chapter II and III, we obtain more insight in the genetic basis of high ethanol tolerance and maximal ethanol accumulation capacity by further dissecting previously identified QTLs by pooled-segregant whole-genome sequence analysis (Pais et al., 2013; Swinnen et al., 2012) and identifying the causative genes responsible for the superior trait phenotypes. In chapter IV, we provide a proof-of-principle for strain improvement by introducing those identified causative alleles for high ethanol tolerance and maximal ethanol accumulation capacity in very high gravity fermentations in an ER segregant with low ethanol tolerance and low ethanol production.

CHAPTER I

Literature overview

1 Biofuels

Since the beginning of this century, there is an increasing interest to use biofuels as an alternative for fossil fuels. These interests are mainly motivated by the increasing oil prices, fast exhaustion of the global oil reserves, concerns about fuel emissions and global warming (Chiari and Zecca, 2011; Mussatto et al., 2010). In 2014, fossil fuels took up 81.1% of the primary energy consumption in the world, of which 64% alone is consumed by the transport sector (IEA, 2016). Therefore, fossil fuels are responsible for a significant amount of pollution in the atmosphere due to the emission of greenhouse gases (GHG) (Escobar et al., 2009; Höök and Tang, 2013). The International Energy Agency (IEA) predicted an increase of one-third in energy demand by 2040, driven primarily by Africa, China, India, the Middle East and Southeast Asia (IEA, 2015). Biofuels provide a way of shifting to low-carbon, non-petroleum, alternative, renewable and sustainable fuels which offer benefits such as increased energy security, by reducing reliance on oil imports, and lowering greenhouse gas emissions. In addition, biofuels can meet development goals by creating new sources of income and employment opportunities in rural areas (IEA, 2011; Prasad et al., 2007; Singh et al., 2010).

Biofuels are defined as liquid and gaseous fuels mainly produced from biomass (plant materials or animal fat) (IEA, 2011). Examples of fuels produced from biomass are bioethanol, biodiesel, methanol, hydrogen and methane (Nigam and Singh, 2011). The use of biofuels as a transport fuel is beneficial by reducing CO_2 build-up due to CO_2 recycling and replacement of fossil fuels (Mussatto et al., 2010). Bioethanol as a transport fuel has a long history. In 1896, Henry Ford produced the first car that could use ethanol as a fuel and in 1908 the Ford Model-T was the first flexible fuel vehicle that could use ethanol, gasoline or a mixture of both (Solomon et al., 2007). After the early 1900s, bioethanol got less attractive due to the increased availability of cheap petroleum-based fuel (Mussatto et al., 2010; Solomon et al., 2007). In the 1970s, the fuel ethanol market revived in Brazil due to the creation of the National Alcohol Program (“Proalcool”), which was a consequence of the global oil crisis and overproduction of sugars at that time (Soccol et al., 2005). Since 2000, the availability of flex-fuel cars combined with growing oil prices have led to the rapid growth of sugarcane and bioethanol production in Brazil. In the United States, the production and use of bioethanol

restarted after 1980 largely due to oversupply of agriculture products (like corn) from the farming sector. Currently, bioethanol is the main biofuel used in the world. In Brazil, all gasoline contains already 27.5% ethanol and the consumers with flex-fuel cars have the choice of adding any higher ethanol blend at the pump in every gas station. Addition of ethanol to gasoline replaces toxic additives, like tetraethyl lead and oil-derived benzene and increases the gasoline octane number (Escobar et al., 2009; Lopes et al., 2016). The United States, followed by Brazil, are world leaders in the production and usage of bioethanol (BP, 2016).

1.1 Classification of biofuels

Biofuels are generally divided into first-, second- and third-generation, depending on the source of biomass and technology used (IEA, 2011) (Fig. 1). First generation (1G) biofuels are commonly produced from food crops with high sugar content, such as sugarcane and corn in the case of bioethanol and vegetable oil in the case of biodiesel, using conventional technologies (Nigam and Singh, 2011). Second generation (2G) biofuels are also known as advanced biofuels (Sims et al., 2010). The feedstocks used for 2G-biofuels are generally non-food crops, but rather lignocellulosic waste materials such as bagasse, straw, forest residues, empty fruit bunches, old paper and cardboard, paper sludge, etc. ... Third generation biofuels refers to biofuels derived from algae (Dragone et al., 2010). In the following paragraphs, we will highlight the classification of biofuels based on the feedstocks used more in detail with emphasis on bioethanol.

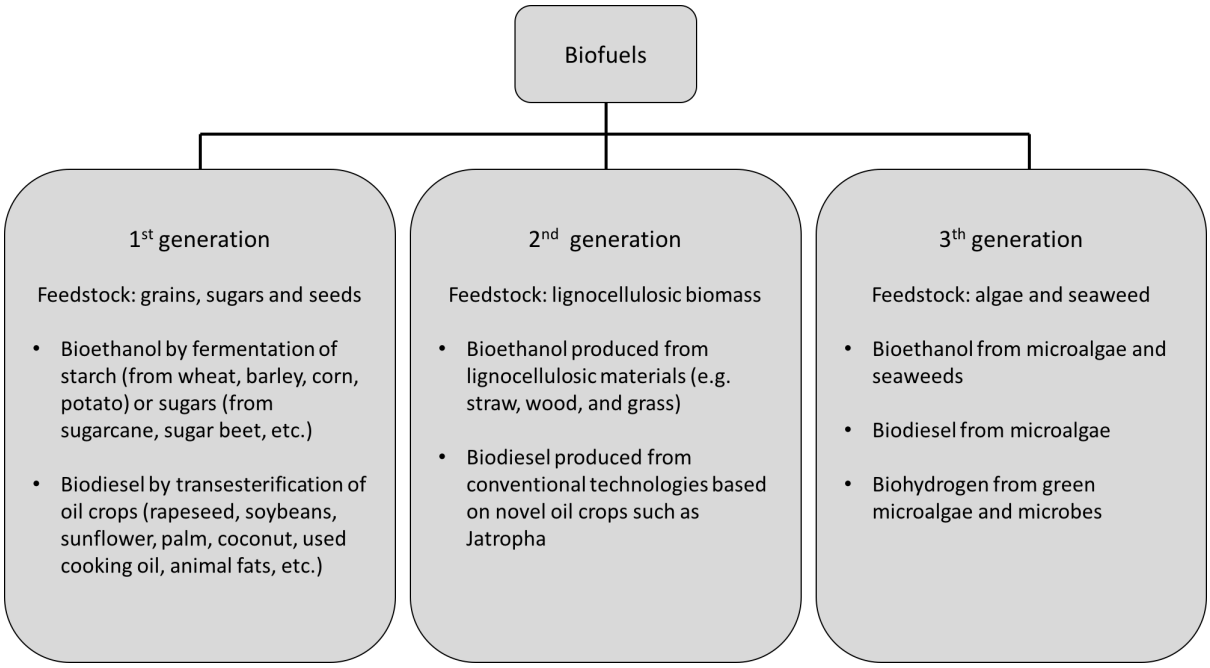


Figure 1: Classification of biofuels. Modified from Nigam and Singh, 2011.

1.1.1 First generation biofuels

First generation biofuels are being produced in significant commercial quantities in a number of countries. Worldwide, bioethanol is the major biofuel, mainly produced by the USA and Brazil from either corn or sugarcane, respectively. In Europe, biodiesel is the main biofuel produced from vegetable oils, while bioethanol is mostly produced from wheat, corn, sugar beet or potato (Havlík et al., 2011).

First generation biodiesel is a fuel composed of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fat (Escobar et al., 2009). Biodiesel is produced by combining oil extracted from seeds with an alcohol through a chemical process known as transesterification. The most common oil crops are rapeseed, soybean, palm and coconut oil (Balat and Balat, 2010). First generation bioethanol, on the other hand, is produced by fermentation of sugar-rich crops (e.g. sugarcane and sugar beet) or starch-rich crops (e.g. wheat, barley, sorghum or cassava). Currently, the main industrial route to produce ethanol is via fermentation performed by micro-organisms such as *Saccharomyces cerevisiae* or *Zymomonas mobilis* (Ho et al., 2014; Lopes et al., 2016). During fermentation, sugars are converted to ethanol, cellular biomass, CO_2 and other by-products. After fermentation,

ethanol can be separated easily from the fermentation broth via a distillation process and further purified from 96% to 100% by molecular sieve (zeolite) technology.

Nevertheless, it is becoming clear that certain first generation biofuels fail to meet specific criteria necessary to be classified reliably as sustainable fuels. First of all, there are concerns about environmental impacts and uncertainties about the potential to reduce GHG emissions. For example, biodiesel production is too expensive in order to reduce GHG emissions, which makes it necessary to look for better alternatives. Second, there are growing concerns about the source of the feedstocks, the competition with usage of fertile land and water for food production and the impact that the feedstock cultivation may have on biodiversity (Gasparatos et al., 2013; Naik et al., 2010; Nigam and Singh, 2011). This has generated the food-versus-fuel debate, in which the increase in biofuel production is considered one of the reasons for rising food prices. All these concerns are currently restricting the increase in first generation biofuel production (Sims et al., 2010).

1.1.2 Second generation biofuels

The perceived disadvantages of the first generation biofuels have stimulated interest in developing second generation (2G) biofuels. The production of 2G biofuels is based on non-food, primarily lignocellulosic biomass, and therefore does not compete with food production. Moreover, this biomass makes up the majority of the cheap and abundant non-food materials of plants and is available in large quantities. The major components of lignocellulosic biomass are the polymers cellulose (an insoluble crystalline polymer consisting of glucose), hemicellulose (non-cellulosic polysaccharide consisting mainly of xylose, arabinose, mannose and galactose) and lignin (an intricate polyphenolic structure) (Hahn-Hägerdal et al., 2006). Lignocellulosic biomass can be divided into three categories: agricultural residues (e.g. crop residues like corn cobs and stalks, sugarcane bagasse, wheat and rice straw); forest residues (e.g. forest thinning, sawdust, etc.), and herbaceous and woody dedicated energy crops (e.g. switchgrass, poplar and miscanthus) (Gupta and Verma, 2015; Sims et al., 2010). Lignocellulosic biomass obtained from energy crops, wood and agricultural residues represents the most abundant global source of renewable biomass (Lin and Tanaka, 2006).

For the production of 2G biofuels, lignocellulosic biomass can be gasified to produce syngas, which in turn can be transformed into liquid hydrocarbons to produce Fisher-Tropsch diesel (FT-diesel), kerosene, methane (Bio-SNG) or dimethyl ether (Bio-DME) (Naik et al., 2010). For production of 2G bioethanol, sugars are extracted by hydrolysis of the lignocellulosic feedstock, after which the sugars are fermented into ethanol. At present, the production of 2G biofuels is not yet cost-effective and some technical and economic issues still have to be overcome.

1.1.3 Third generation biofuels

For the production of third generation (3G) biofuels, the feedstock originates from marine biomass (e.g. algae). Algae are uni- and multicellular photoautotrophs, which absorb energy from sunlight for growth and production of intracellular compounds like carbohydrates, lipids and proteins (Costa and de Morais, 2011). They can be classified into two groups based on size and morphology: microalgae and macroalgae (seaweed). Algae have a very high photosynthetic efficiency compared to most terrestrial crops (Alaswad et al., 2015). The use of algal biomass for biofuel production has numerous advantages: high growth rates; growth throughout the whole year; sequestration of CO_2; low water consumption for cultivation; no need of herbicides or pesticides; growth in harsh conditions like saline, brackish coastal seawaters and therefore they do not interfere with conventional agriculture. Nevertheless, there are also several disadvantages, such as complex composition and very high cultivation and harvesting costs compared to conventional crops (Behera et al., 2014; Brennan and Owende, 2010).

Microalgae have in general a very high oil content, up to 80% by weight of their dry biomass. This makes it an attractive feedstock for biodiesel production. The microalgae *Kirchneriella lunaris*, *Ankistrodesmus fusiformis*, *Chlamydocapsa bacillus*, and *Ankistrodesmus falcatus* have been preferred species for biodiesel production (Behera et al., 2014). While microalgae such as *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Spirulina* have been used for bioethanol production because of their high content of starch and glycogen (Harun et al., 2014). Macroalgae, such as *Laminaria*, *Saccorhiza*, *Alaria* and *Gelidium amansii* can also be used for bioethanol production. This last category produces high levels of polysaccharides such as starch and cellulose and does not need structural biopolymers like lignin and

hemicellulose. This simplifies the production process of bioethanol, since there is no need for the cumbersome pretreatment of the biomass. The fermentable sugars can be converted to bioethanol by a suitable ethanol producer, however some algae can produce ethanol themselves during dark fermentation (John et al., 2011). Although, algae have the potential to be converted into different types of renewable biofuels including bioethanol, biodiesel, biogas and biohydrogen, many technical and scientific barriers still have to be overcome before this can become a commercial reality.

1.2 Bioethanol as a renewable feedstock for bio-based chemicals

Bioethanol is mainly used as a transportation fuel, while only 18% of bioethanol is used for non-fuel applications such as solvents in the cosmetics and pharmaceuticals industry, detergents, inks and coatings (Harmsen et al., 2014). Nowadays, there is growing interest in the use of bioethanol as a renewable building block for the production of bio-based plastics. Ethylene is one of the most important chemical building block for polymers such as polyethylene (PE), polyethylene terephthalate (PET), polyvinylacetate (PVA), polyvinylchloride (PVC), and many others. Although ethylene is mainly produced in the petrochemical industry by steam cracking of naphta, several companies (Braskem, Solvay and Dow Chemical-Mitsui Chemicals) have succeeded in commercializing bioethylene production by catalytic dehydration of bioethanol (Mohsenzadeh et al., 2017). Furthermore, production of green solvents by esterification of ethanol with a carboxyl acid such as ethyl lactate (Vertec Biosolvents) and ethyl acetate (Zeochem) has also been commercialized (Choi et al., 2015). Nonetheless, this non-fuel market will only slowly grow unless there is a breakthrough in 2G bioethanol production, a tremendous rise in the crude oil price or important developments in regulatory policies.

1.3 Bioethanol production process

1.3.1 First generation bioethanol production process

Besides the difference in feedstock (see 1.1.1), there are also large differences in the production process between the two main ethanol producing countries in the world, Brazil and USA. First, in Brazil, the yeast cells are recycled after each fermentation, while in the USA, the yeast is not recycled due to the high concentrations of solids in the fermentation media. Second, fermentation times are much shorter in Brazil (6-12h) compared to the USA (54-72h) because of the much higher yeast pitching rate. On the other hand, corn fermentation can reach a higher ethanol concentration (12-18% v/v) compared to sugarcane fermentations (7-12% v/v) (Lopes et al., 2016). The fermentation of starch-rich biomass is more complex because of the hydrolysis of starch into fermentable sugar by enzymes such as amylase and glucoamylase. This makes the energy requirement of starch-based ethanol production significantly greater than that of sugar-based ethanol production (Galbe et al., 2007). Third, co-products of the corn fermentation process like distillers dried grains and solubles (DDGS) can be sold as protein-rich animal feed with a relatively high market value due to its nutritional characteristics (Martinez-Amezcua et al., 2007). Brazilian distilleries, on the other hand, generate potassium-rich vinasse as co-product that is used for sugarcane fertilization-irrigation, reducing costs on fertilizers (Lopes et al., 2016).

1.3.2 Second generation bioethanol production process

The conversion of lignocellulosic biomass to ethanol requires additional steps in the production process compared to first generation bioethanol (Fig. 2). During the 2G bioethanol production process, a first step of size reduction is necessary. This can be done by chipping, grinding or milling. This size reduction step is necessary to liberate cellulose and hemicellulose from their complex with lignin. In the next step of the pretreatment, lignin and hemicellulose are removed from the cellulose fraction, this allows better accessibility of enzymes and/or chemicals to the cellulose. Steam explosion (physical-chemical pretreatment) is the most common pretreatment, the chipped biomass is treated with high pressure steam, followed by sudden decompression to atmospheric pressure. As a result, the lignocellulosic matrix separates into individual fibres. Another method is chemical pretreatment of the biomass by chemical agents such as ozone, acids, alkalis, peroxide and organic solvents. Both H_2SO_4 and

HCl have been used preferably as chemical agents for biomass pretreatment (Alvira et al., 2010). A negative consequence of the pretreatment is the release of various compounds that are inhibitory to the fermentation of micro-organisms. In general, there are three major groups of inhibitors: furan derivatives (HMF and furfural), weak acids (mainly acetic acid) and phenolic compounds (Almeida et al., 2007).

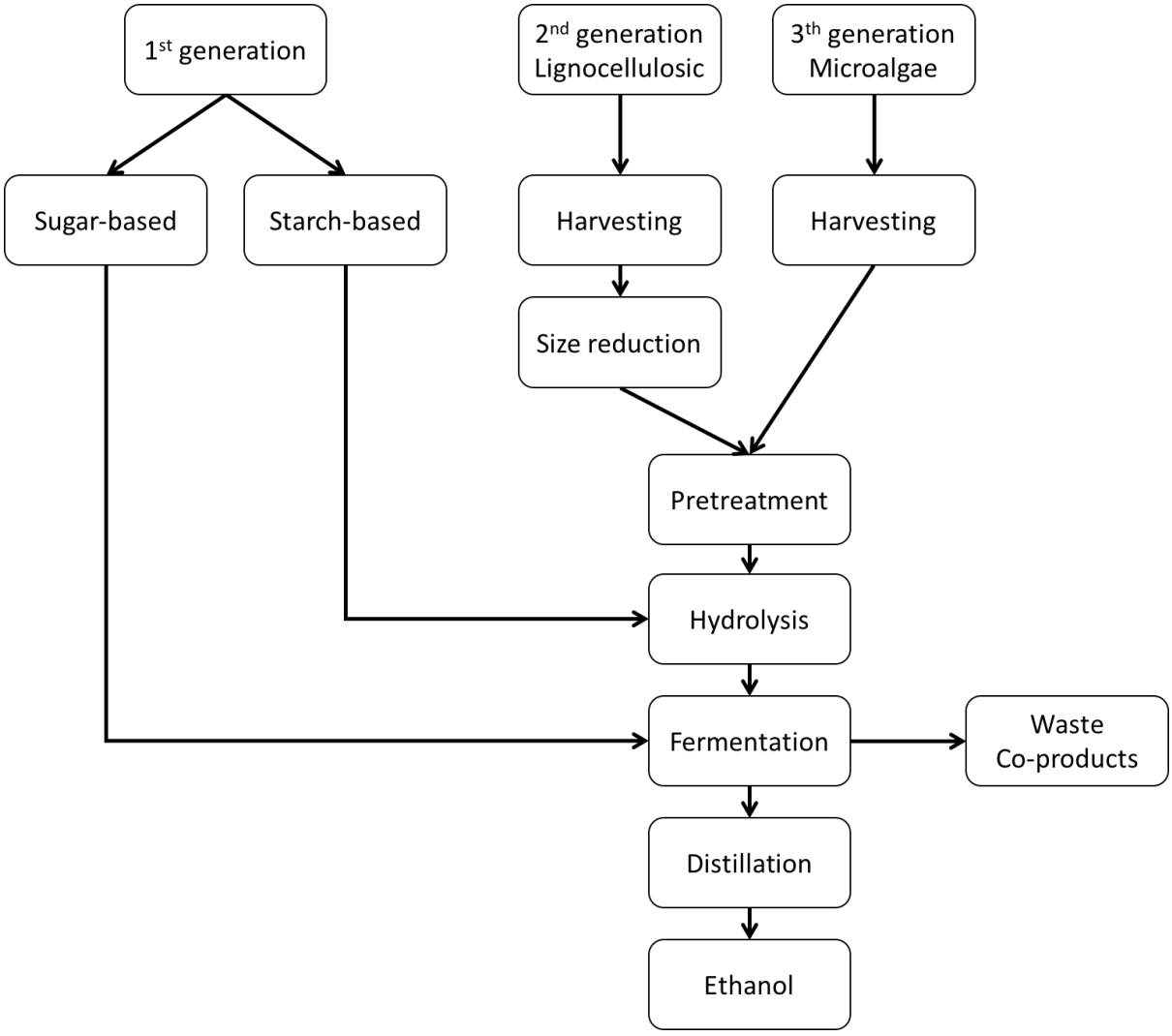


Figure 2: Overview of the bioethanol production processes with different feedstocks. Modified from Baeyens et al., 2015.

In the next step, acid or enzymatic hydrolysis is necessary to obtain free sugars for fermentation. Enzymes that break up the β-1-4 glycosidic bonds of cellulose are called cellulases. They are mainly produced by fungi such as *Trichoderma reesei*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Baeyens et al., 2015). The optimum temperature of cellulase activity is generally above 50°C, while yeast strains have an optimal growth and fermentation temperature in the range of 30-37°C. Because of this difference in optimal conditions "separate hydrolysis and fermentation processes" (SHF) were first designed. Since cellulases are strongly inhibited by the cellulose hydrolysis products (glucose and glucose oligomers) the "simultaneous saccharification and fermentation" (SSF) process was designed later on in order to overcome this inhibition. During SSF, enzymatic hydrolysis and fermentation are combined to keep the glucose concentration low. As a consequence, there is reduced performance of the enzymatic cocktail due to the difference in optimum temperature for hydrolysis (45-50°C) and fermentation (28-35°C). Although sugars are more inhibitory for the enzymes than ethanol, SSF can reach higher rates, yields and ethanol concentrations than SHF (Koppram and Olsson, 2014). Another advantage of SSF is the much lower risk of bacterial contamination due to the fact that the free sugar level is continuously kept low. In the last step, the ethanol produced is recovered by distillation and molecular sieve (zeolite) treatment from the fermentation broth.

1.3.3 Very high gravity fermentation

Rapid fermentation and high final ethanol concentrations are very important for the bioethanol production industry. One way to improve the overall productivity of the bioethanol production process is the implementation of very high gravity (VHG) fermentation. VHG fermentation is defined as the fermentation of mashes containing 27g or more of dissolved solids per 100g mash (Thomas et al., 1993). The advantages of VHG fermentation include considerable water savings, a higher ethanol yield (15 - 18% v/v), reduced labor and capital costs as well as minimized bacterial contamination (Thomas and Ingledew, 1995), thus minimizing the production costs also because of energy savings (Puligundla et al., 2011).

The major disadvantage of VHG fermentations is the high viscosity in the fermentor due to the high sugar concentration. High viscosity can compromise the ethanol production process. It can hamper the solid-liquid separation, which results in incomplete hydrolysis of starch and

low fermentation efficiency (Zhang et al., 2010). To lower the viscosity during the VHG starch/glucose based bioethanol process, pectine-degrading and proteolytic enzymes and/or other enzymes, such as xylosidases, cellulases and xylanases, are used (Devantier et al., 2005; Wang et al., 2007). This viscosity challenge is more pronounced in lignocellulosic processing compared to VHG starch fermentation (Koppram et al., 2014). An additional disadvantage is the formation of inhibitory compounds during the pretreatment, which influences both the enzymes and micro-organisms more severely at high substrate concentrations. In addition, VHG technology also leads to increased stress conditions for yeast cells, which can result in sluggish and stuck fermentations, hence decreasing the ethanol production efficiency.

There are a number of causes for fermentation arrest: nitrogen availability, sugar concentration, fermentation temperature and increasing ethanol concentrations (Bauer and Pretorius, 2000; Bisson, 1999). To overcome this problem, yeast strains have to be resistant to multiple stresses, like high osmotic pressure, nutrient limitation, elevated ethanol concentration and the anaerobic conditions which are common in the large-scale bioreactors (Patkova et al., 2000). In summary, VHG fermentation of lignocellulosic biomass still requires further optimization.

1.3.4 Micro-organisms for bioethanol production

The yeast *S. cerevisiae* is commonly used to convert C6 sugars into ethanol (Bai et al., 2008). *Zymomonas mobilis* has also been proposed for that purpose. Both micro-organisms possess high ethanol tolerance but unfortunately are unable to ferment C5 sugars.

Z. mobilis, an anaerobic gram-negative bacterium, produces ethanol from glucose via the Entner-Doudoroff (ED) pathway, followed by reduction of pyruvate to ethanol. During conversion of glucose in the ED pathway, only one molecule of ATP is produced. As a consequence, less biomass is produced compared to *S. cerevisiae* and more carbon is funnelled into ethanol production. This leads to a higher ethanol yield, 97% of the theoretical yield of ethanol to glucose, compared to 90-93% achieved by *S. cerevisiae* (Sprenger, 1996). The advantages of using *Z. mobilis* over *S. cerevisiae* are the following: higher sugar uptake and ethanol yield, less biomass production, high temperature tolerance and high ethanol tolerance due to the fact that the plasma membrane contains hopanoids, which are similar

to eukaryotic sterols (Baeyens et al., 2015). Disadvantages of *Z. mobilis* are the following: a very specific substrate spectrum (only D-glucose, D-fructose and sucrose), cannot ferment C5-sugars such as xylose and arabinose, the biomass is not commonly accepted as animal feed, the organism cannot tolerate toxic inhibitory compounds, such as acetic acid and phenolic compounds from lignocellulosic biomass and bacterial contamination is very difficult to combat with a bacterium as host (Baeyens et al., 2015; Bai et al., 2008). Currently, the presence of toxic inhibitors in the lignocellulosic hydrolysates still compromises efficient fermentation by engineered *Z. mobilis*, which hampers their industrial application (Yi et al., 2015).

In yeast, glycolysis or the Embden-Meyerhof-Parnas pathway is the main metabolic pathway involved in ethanol fermentation. During glycolysis, one molecule of glucose is metabolized into two molecules of pyruvate. In a next step pyruvate is further reduced to ethanol with CO_2 release (Bai et al., 2008). Due to the fact that glycolysis results in production of two ATPs, which drive the biosynthesis of yeast cells, ethanol production and yeast proliferation are tightly coupled. Glycolysis will be interrupted when there is intracellular accumulation of ATP because of ATP inhibition of phosphofructokinase, one of the important enzymes of glycolysis (Bai et al., 2008).

During the production process of bioethanol, yeast cells suffer from various stresses such as osmotic stress, nutrient deficiency, high temperature, contamination, inhibitory compounds such as acetic acid and furfural, and ethanol accumulation (Fig. 3). These stresses often lead to reduced yeast viability and fitness, hence lowering the final ethanol yield.

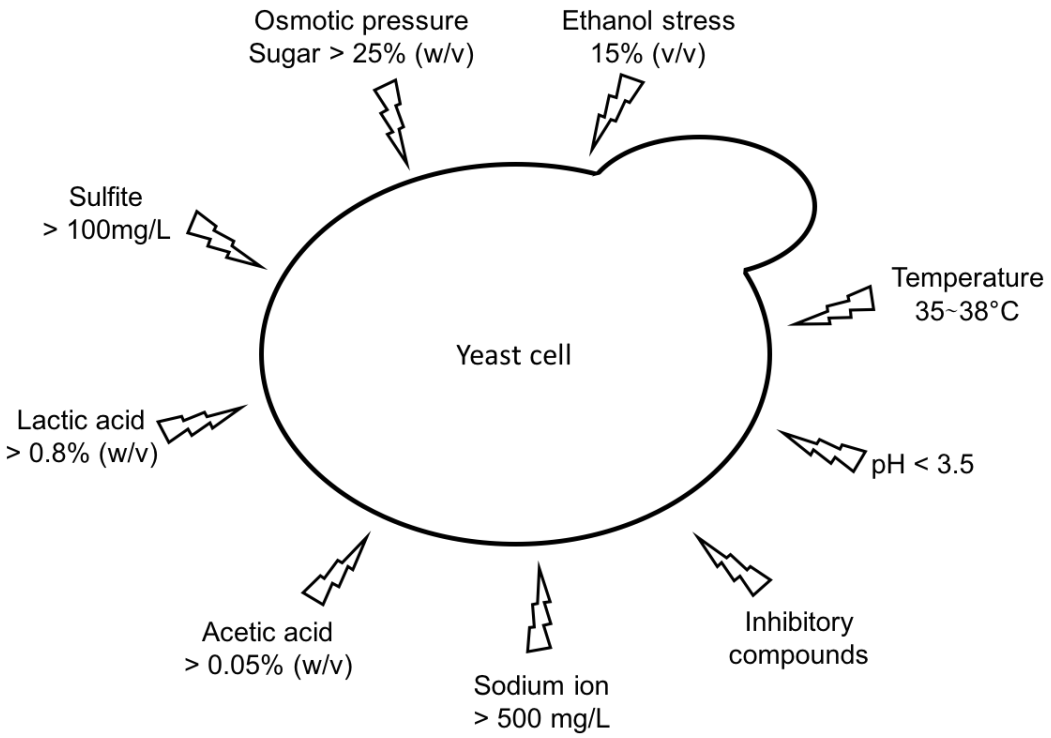


Figure 3: Potential environmental stresses on S. cerevisiae during industrial ethanol fermentations. Modified from Bai et al., 2008.

Currently, *S. cerevisiae* is the organism of choice for bioethanol production (Basso et al., 2008). However, during first and second generation bioethanol production, the yeast has to overcome a variety of stresses during the fermentation process that reduce the fermentation rate and ethanol yield. These include ethanol stress, osmotic stress, temperature stress and inhibitor stress. Different non-conventional yeasts and bacteria are under investigation for their tolerance to these stresses (Radecka et al., 2015). *Candida shehatae*, *Pichia stipitis* and *Escherichia coli* are able to use both pentose and hexose sugars, but have low tolerance to ethanol. The thermophilic yeast *Kluyveromyces marxianus* is able to tolerate temperatures up to 50°C, which could be beneficial during SSF. However, it has a low ethanol tolerance and a poor xylose fermentation capacity (Baeyens et al., 2015). *Zygosaccharomyces rouxii,* on the other hand, can grow in the presence of high sugar and salt concentrations, which is important in very high gravity fermentation, in which there is high osmotic pressure (Radecka et al., 2015). Another interesting non-conventional yeast is *Dekkera bruxellensis,* which has an ethanol tolerance comparable to *S. cerevisiae* and shows better tolerance to inhibitors, which is of interest for second generation bioethanol production (Ruyters et al., 2015). All these non-conventional yeast species can also give us more insight in the molecular basis of high tolerance to those stresses in order to further improve industrial strains of *S. cerevisiae* for these properties.

Genetic and evolutionary engineering of *S. cerevisiae* has resulted in a yeast strain that gained the capacity to ferment glucose and pentose sugars simultaneously. Demeke and colleagues (2013a) succeeded in developing the an industrial *S. cerevisiae* strain that converts D-xylose into ethanol with a very high specific fermentation rate and a high ethanol yield. Additionally, Demeke and colleagues (2013b) developed industrial yeast strains that combine efficient D-xylose utilization and high inhibitor tolerance and therefore show high potential for use in second generation bioethanol production from lignocellulosic hydrolysates.

2 The cellular impact of ethanol stress on *S. cerevisiae*

Saccharomyces cerevisiae is one of the most effective bioethanol producing micro-organisms. One of the main advantages of this micro-organism is its high ethanol tolerance, high ethanol productivity and high inhibitor tolerance (Olsson and Nielsen, 2000). One of the most common stresses that yeast cells encounter during VHG fermentation is the high concentration of ethanol. Yeast cells have developed different mechanisms to counteract the disastrous effects of high ethanol concentrations produced during fermentation in sugar-rich environments in nature. The main targets of ethanol are the cellular membranes and cell wall (Jones and Greenfield, 1987), proteins like glycolytic enzymes and alcohol dehydrogenase (ADH) (Nagodawithana and Steinkraus, 1976), the vacuole and the endoplasmic reticulum (Meaden et al., 1999; Stanley et al., 2010a). In general, *S. cerevisiae* is highly ethanol tolerant compared to other micro-organisms. However, high levels of ethanol also inhibit cell growth and reduce cell viability in *S. cerevisiae*, thereby limiting fermentation productivity and ethanol yield (Aguilera et al., 2006; Ansanay-Galeote et al., 2001; Kasavi et al., 2012). In the last decades, many research groups have intensively studied the ethanol-tolerance mechanisms in *S. cerevisiae* (Ding et al., 2009; Ma and Liu, 2010b; Stanley et al., 2010a). Improving our understanding of the cellular impact of ethanol toxicity and how the cell responds to ethanol stress can facilitate the development of strategies for improving ethanol tolerance and fermentation productivity. However, ethanol tolerance is a complex, polygenic trait of which the genetic basis and the interplay between multiple alleles are not yet understood. In this section, we describe the impact of ethanol toxicity on the yeast cell and how the cell responds to it.

2.1 Ethanol stress and damage to cellular membranes

2.1.1 Plasma membrane composition and ethanol damage

Cellular membranes are the primary target of the toxic effect of ethanol in yeast. Ethanol interferes with membrane structure and composition, hereby increasing membrane fluidity and thus directly affecting the imbedded membrane proteins. Ethanol interacts with the phospholipid head group of the lipid bilayer, consequently affecting the chemical-physical

properties and the functions of the membrane (Weber and de Bont, 1996). Low ethanol concentrations will fluidize the membrane by increasing the lipid head group mobility. At higher ethanol concentrations, the fatty acid chains will cross the lipid bilayer midplane whereby ethanol shields the fatty acid methyl groups from the water phase. As a consequence, the membrane will reduce in thickness when it shifts to the interdigitated phase (Fig. 4) (Vanegas et al., 2012). Membrane thinning by ethanol has a profound effect on integral membrane protein conformation and function due to exposure of hydrophobic amino acids to an aqueous environment, a phenomenon known as hydrophobic mismatch (Killian, 1998; Lee, 2004). To survive under ethanol stress, yeast cells change their membrane composition to antagonize the membrane fluidization resulting in stabilization of the plasma membrane structure (Swan and Watson, 1998; You et al., 2003). This is done by changing the unsaturated fatty acid (UFA) index as well as the ergosterol levels in the membranes (Henderson and Block, 2014). Furthermore, ethanol enhances the passive proton influx through the plasma membrane, leading to dissipation of the membrane potential and proton gradient, which results in the inhibition of nutrient uptake that depends on the electrochemical gradient (Madeira et al., 2010). Several studies suggest that specific amino acids (see 2.1.2) and inositol also enhance ethanol tolerance, probably by enhancing membrane stability in the presence of ethanol (Hu et al., 2005; Kelley et al., 1988; Takagi et al., 2005).

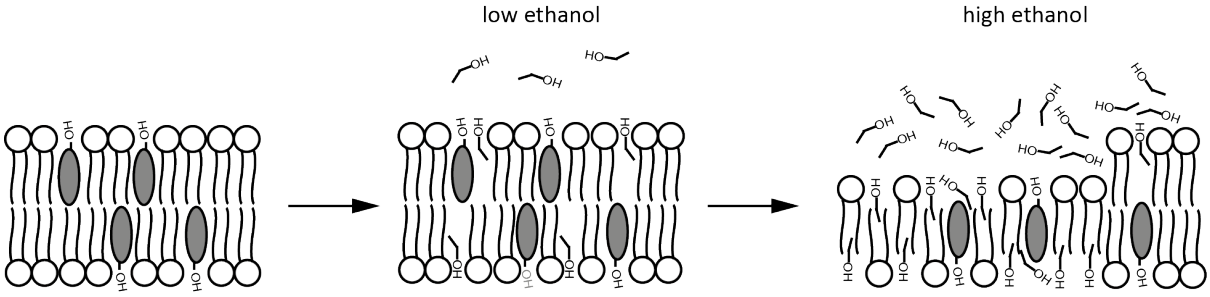


Figure 4: Illustration of the effects of low and high ethanol concentrations on the membrane. Sterols are shown as grey ellipses. Modified from Vanegas et al., 2012.

2.1.1.1 Phospholipids

The cellular membrane of yeast is mainly composed of phospholipids, glycosphingolipids, ergosterol and proteins. Phospholipids consist of a glycerol-3-phosphate backbone with two esterified fatty acid chains and a hydrophilic head group consisting primarily of choline, ethanolamine, inositol or serine (Daum et al., 1998). The most common fatty acids in yeast are palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and the unsaturated fatty acids (UFAs) palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$). Ergosterol is the predominant sterol in yeast, followed by lanosterol and squalene (Gaspar et al., 2007). Due to the amphiphilic character of phospholipids, they spontaneously form asymmetric lipid bilayers with embedded integral and attached peripheral proteins.

Many studies report that yeast cells increase their content of monounsaturated fatty acids in the cellular membranes when grown in the presence of ethanol (Alexandre et al., 1994; Sajbidor et al., 1995; Wang et al., 2015). Oleic acid is the most important unsaturated fatty acid in these conditions apparently aimed at overcoming the inhibitory effect of ethanol on yeast cells (Ding et al., 2009; You et al., 2003). On the other hand, both UFAs, palmitoleic acid and oleic acid, are formed by the same oxygen- and NADH-dependent desaturase Ole1. However, reports on transcription dynamics in response to ethanol stress do not show a significant difference in *OLE1* mRNA expression (Chandler et al., 2004; Ma and Liu, 2010a). One possibility is that under ethanol stress, UFA biosynthesis is regulated at the level of mRNA stability and translation instead at transcriptional level (Martin et al., 2007). Wang and colleagues (2015) showed that after evolutionary engineering, ethanol-adapted *S. cerevisiae* cells had a higher percentage of UFAs and a lower percentage of saturated fatty acids due to higher transcriptional expression of *OLE1.* Higher concentrations of UFAs are known to result in higher membrane fluidity, counteracting the effect of other components like ergosterol, which make the cell membranes more rigid (Ding et al., 2009).

2.1.1.2 Ergosterol

Ergosterol is a second important component in the yeast cell membranes. It regulates the balance of lipids and proteins in the membranes (Shobayashi et al., 2005). Several studies suggest that ergosterol plays an important role in membrane stability and survival under ethanol stress, by making the cellular membrane more rigid. In this way, ergosterol

antagonizes the membrane fluidization effect caused by high ethanol concentrations (Alexandre et al., 1994; Swan and Watson, 1998). Yeast strains with a higher ergosterol content in the membranes are found to have a higher ethanol tolerance (Aguilera et al., 2006; Swan and Watson, 1998). Deletion mutants in different genes involved in ergosterol biosynthesis showed defective growth under ethanol stress (Auesukaree et al., 2009; Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). On the other hand, the expression of genes involved in ergosterol biosynthesis is down-regulated during ethanol stress (Alexandre et al., 2001; Chandler et al., 2004; Ma and Liu, 2010a).

In the literature, as is also clear from the data mentioned above, there is still controversy about the function of the membrane lipid composition under conditions of ethanol stress. Ethanol affects the fluidity of the membranes, hereby compromising their integrity and consequently loosening the membrane structure (Ma et al., 2013). It is remarkable that ethanol also causes an increase in UFAs, since this further increases the membrane fluidity. On the other hand, increased ergosterol levels would counteract the effect of increased UFA levels (Ding et al., 2009). Wang and coworkers (2015) have proposed that increased levels of ergosterol might maintain the integrity of the cell membrane through maintenance of the crosslinking between different membrane components. On the other hand, maintaining membrane fluidity by increasing UFA concentrations might be essential to preserve cellular membrane dynamics in order to adapt better to the ethanol stress and maintain cell viability (Kajiwara et al., 2000).

2.1.2 The role of amino acids

In the literature, some amino acids have been reported to contribute to ethanol tolerance. In a study by Hu and coworkers (2005), yeast cells were exposed to an ethanol solution (20% v/v) supplemented with three amino acids: methionine, isoleucine and phenylalanine. These cells exhibited a higher cell viability compared to cells grown in medium without added amino acids. This study showed that the increase in ethanol tolerance was due to the incorporation of the supplementary amino acids into the plasma membrane, causing enhanced ability to counteract the fluidizing effect of ethanol. Tryptophan is another amino acid that is believed to contribute to ethanol tolerance. Deletion of genes involved in tryptophan biosynthesis

caused a decrease in ethanol tolerance (Hirasawa et al., 2007; Yoshikawa et al., 2009). Overexpression of tryptophan biosynthesis and permease genes as well as supplementation of tryptophan in the medium, on the other hand, enhanced ethanol tolerance of yeast (Ma and Liu, 2010b). In yet another study it was shown that accumulation of L-proline in yeast cells could improve ethanol tolerance (Takagi et al., 2005). L-proline is a known osmoprotectant that helps to protect cells from freezing, desiccation or oxidative stress by stabilization of proteins and membranes as well as inhibition of protein aggregation during the (re)folding process (Morita et al., 2003; Terao et al., 2003). Takagi and coworkers (2005) constructed a yeast strain that accumulated intracellular L-proline and showed greater cell viability when grown in the presence of ethanol compared to its parent strain. Recently, investigators found that supplementation of arginine in the medium had a protective function on yeast cells in the presence of ethanol. By overexpression of *ARG4* or deletion of *CAR1,* genes involved in arginine biosynthesis and degradation, respectively, cells accumulated more intracellular arginine, which made them more tolerant to ethanol compared to the control strains (Cheng et al., 2016). Further research showed that during ethanol stress, the cell wall of strains with higher arginine content was thicker compared to the control. This result indicated that arginine plays a role in maintenance of cell wall integrity under ethanol stress. Moreover, membrane damage caused by ethanol was inversely proportional to the intracellular arginine content, which indicates that arginine has a protective function on the plasma membrane. In addition, it was found that arginine could reduce cellular oxidative stress caused by ethanol by lowering the level of reactive oxygen species (ROS) generated, which resulted in higher ethanol tolerance (Cheng et al., 2016).

2.1.3 Intracellular pH and H^+-ATPases

Ethanol affects membrane integrity of the yeast cell, which leads to an increase in membrane permeability, resulting in passive proton influx and acidification of the cytosol (Leao and Van Uden, 1984). An increase in passive proton influx will dissipate the proton electrochemical gradient across the plasma membrane which is important for driving secondary transport of nutrients (Cartwright et al., 1987; Rosa and Sa-Correia, 1991). The plasma membrane proton-pumping ATPases, encoded by *PMA1* and *PMA2,* are the major proteins responsible for maintenance of the electrochemical gradient across the membrane, hence controlling cytosolic pH. Likewise, transport of intracellular protons into the vacuole, mediated by the

vacuolar H^+-ATPase (V-ATPase), is important to maintain intracellular pH homeostasis and therefore counteracting ethanol stress (Inoue et al., 2005). Since the extrusion of protons by the H^+-ATPases is highly energy demanding, consuming up to 20-40% of cellular ATP depending on the conditions, it is a strictly controlled process (Morsomme et al., 2000).

The plasma membrane H^+-ATPase, which is a major factor in determining the strength of the proton electrochemical gradient, is non-competitively inhibited by higher ethanol concentrations (Cartwright et al., 1987). It has been shown that changes in the phospholipid head-group orientation as well as fatty-acyl chain length and degree of unsaturation due to stress modify the activity of the yeast plasma membrane H^+-ATPase (Stadtlander et al., 1982). Whereas addition of ethanol in an *in vitro* ATPase assay reaction causes partial inhibition, yeast cells exposed *in vivo* to sub-lethal ethanol levels dramatically increase the activity of their H^+-ATPases (Rosa and Sa-Correia, 1991). Fernandes and Sa-Correia (2003) showed that the expression level of both *PMA1* and *PMA2* are increased when yeast cells are cultivated in ethanol-based medium. (Monteiro et al., 1994). Lee and co-workers (2017) showed that overexpression of *PMA1* confers enhanced tolerance to ethanol, ROS, acetic acid and other weak acids. Moreover, Lam and co-workers (2014) showed that overexpression of the potassium importer *TRK1* as well as *PMA1* in a laboratory strain greatly enhances ethanol tolerance and the performance of the strain in VHG fermentation.

Likewise, deletion of a number of genes associated with V-ATPases, including genes encoding structural components of the V-ATPase as well as genes involved in the assembly of the V-ATPase, caused a growth defect during ethanol stress. Also, genes involved in the vacuolar protein sorting machinery and vacuolar membrane structure and function are required for ethanol tolerance (Auesukaree et al., 2009; Fujita et al., 2006; Teixeira et al., 2009; Yoshikawa et al., 2009). Researchers found that, when V-ATPase activity is lost in organelles, it triggers ubiquitination and endocytosis of Pma1. This possibly suggests that the V-ATPase and the plasma membrane H^+-ATPase are both important and dependent on each other to regulate the intracellular pH (Charoenbhakdi et al., 2016; Smardon and Kane, 2014). Recently, it was shown that ethanol sensitivity of mutants lacking V-ATPase activity results from increased intracellular ROS accumulation and a defect in cell wall remodelling rather than severe

cytosolic acidification. It could, however, not be excluded that the increased oxidative stress was a consequence of chronic cytosolic acidification (Charoenbhakdi et al., 2016).

2.1.4 Stabilisation and repair of cell components during ethanol stress

Ethanol stress not only leads to changes in the plasma membrane and the intracellular pH, but can also cause damage to other essential cellular components such as nucleic acids and proteins. The two most common cellular responses to ethanol stress are the accumulation of trehalose and the expression of heat shock proteins (HSPs), that are thought to prevent ethanol damage to cellular components.

2.1.4.1 Trehalose

Trehalose is one of the reserve carbohydrates in the yeast *S. cerevisiae*. This molecule is synthesized in a two-step reaction involving trehalose-6-phosphate synthase, Tps1, which catalyses the conversion of UDP-glucose and glucose-6-phosphate to trehalose-6-phosphate, and trehalose-6-phosphate phosphatase, Tps2, which catalyses the final step of trehalose biosynthesis, dephosphorylation of trehalose-6-phosphate to trehalose (Bell et al., 1998). Trehalose is hydrolysed into glucose by two neutral trehalases, Nth1 and Nth2, or by an acid-vacuolar trehalase, Ath1 (Jules et al., 2008). Trehalose is a non-reducing disaccharide of glucose, that has two major functions, energy reserve and stress protectant. Trehalose synthesis is increased during stationary phase (Francois et al., 1991). The accumulated trehalose can, on the one hand, serve as carbon reserve for the yeast cell, next to glycogen (Jules et al., 2008; Shi et al., 2010; Van Dijck et al., 1995). The stress-protecting function, on the other hand, includes (i) preservation of plasma membrane integrity by substituting water molecules and binding to the polar head groups of phospholipids and (ii) functioning as a co-chaperone by stabilizing proteins in their native state preventing damage as well as by suppressing the aggregation of misfolded proteins (Singer and Lindquist, 1998; Wiemken, 1990).

In *S. cerevisiae,* trehalose is accumulated in response to different stress conditions, such as ethanol (Attfield, 1987; Divate et al., 2016; Sharma, 1997), freezing (Nakamura et al., 2009), heat (De Virgilio et al., 1994), saline (Mahmud et al., 2009), acetic acid (Yoshiyama et al., 2015) and other environmental stresses (Mahmud et al., 2010). All these studies claim that the

higher the trehalose content in the yeast cells, the higher their tolerance against environmental stress. Ogawa and co-workers (2000) revealed that cells unable to accumulate trehalose showed retarded growth in the presence of high ethanol concentrations. Moreover, several genes involved in trehalose metabolism are up-regulated under ethanol stress (Alexandre et al., 2001; Chandler et al., 2004). Co-induction of trehalose synthesis and degradation genes appears to be important in order to balance optimal concentrations of trehalose and energy required for proper cell functionality (Ma and Liu, 2010b).

Many laboratory yeast strains have been genetically engineered to explore the correlation between trehalose and ethanol stress. (Jung and Park, 2005; Kim et al., 1996; Mahmud et al., 2010). Recently, industrial yeast strains were genetically engineered to accumulate more trehalose by overexpression of *TPS1* and deletion of one of the degrading enzymes *NTH1* or *ATH1*. Those engineered strains showed higher ethanol tolerance and achieved higher ethanol yields in the presence of high initial glucose concentrations compared to their wild type strains (Divate et al., 2016; Wang et al., 2014). However, doubt about this presumed stress-protecting function of trehalose has been raised based on some controversial opinions about the exact role of trehalose in ethanol tolerance (Alexandre et al., 1998; Gomes et al., 2002; Petitjean et al., 2015). Petitjean and co-workers (2015) elucidated the role of trehalose and/or Tps1 under different stress conditions, by constructing a yeast strain that expressed a catalytically inactive variant of Tps1 unable to synthesise trehalose-6-phosphate and thus trehalose. They showed that yeast tolerance to various stresses, including ethanol stress, was largely independent of the trehalose accumulated, but relied on the Tps1 protein itself. They proposed that Tps1 is an example of a “moonlighting protein”, which is characterised by the ability to perform completely unrelated functions to the regular function. Apparently, Tps1 bears a regulatory function, independent from its enzymatic function in trehalose synthesis. Tps1 might have an important function in preventing energy depletion through regulation of energy homeostasis.

2.1.4.2 Heat shock proteins

Heat shock proteins (HSPs) belong to a family of protective proteins that are induced in response to stress stimuli in yeast cells (Piper, 1995). Many of these HSPs are molecular chaperones and mediate the correct folding of other proteins to prevent further damage to the cell (Craig et al., 1993). Most of the HSPs induced by ethanol seem to be identical to those induced by heat shock. There is also evidence that many changes induced by ethanol stress are identical to those caused by heat stress, such as membrane disordering, protein denaturation, trehalose accumulation, plasma membrane H^+-ATPase activity and other changes (Piper, 1995). Increase in ethanol tolerance can be induced by pre-exposure to heat shock. However, pre-exposure to ethanol stress leads to hypersensitivity of cells to heat (Van Uden, 1983; Watson and Cavicchioli, 1983).

Different researchers observed that the threshold ethanol concentration for strong induction of the transcription of *HSP* genes is between 4 and 10% (Kubota et al., 2004; Piper et al., 1994). In *S. cerevisiae,* global gene expression analysis during ethanol stress identified at least 10 upregulated *HSP* genes, mainly *HSP12*, *HSP26*, *HSP30*, *HSP31*, *HSP32*, *HSP42*, *HSP78*, *HSP82*, *HSP104*, and *HSP150.* Additionally, other genes encoding chaperones also showed higher expression levels during ethanol stress (Alexandre et al., 2001; Chandler et al., 2004; Ma and Liu, 2010a; Marks et al., 2008). Studies with *HSP* deletion mutants also supported a direct contribution of HSPs to ethanol tolerance (Sales et al., 2000; Sanchez et al., 1992). These results indicate that protein unfolding might be an important aspect of ethanol toxicity and that activation of HSPs is necessary to counter the damaging effect of ethanol on protein structure.

2.1.4.3 Induction of antioxidant proteins

Ethanol stress is related to the formation of reactive oxygen species (ROS). When yeast cells are exposed to ethanol stress, harmful ROS production is dramatically increased. ROS can be neutralized in yeast by both non-enzymatic and enzymatic antioxidant defense mechanisms (Costa et al., 1997). In response to ethanol stress, yeast cells increase the expression of various antioxidants such as catalase T (product of *CTT1*) and the mitochondrial superoxide dismutase, also called manganese dependent (MnSOD, product of *SOD2*) (Alexandre et al., 2001; Pereira et al., 2001). These two antioxidants play an important role in ethanol tolerance

preventing damage to mitochondrial DNA, lipids and proteins caused by ROS such as superoxide (O_2^-), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). Other antioxidants, such as glutathione and cytoplasmic CuZn superoxide dismutase, play a minor role during ethanol stress (Moradas-Ferreira et al., 1996; Piper, 1995). Costa and co-workers (1997) showed that loss of superoxide dismutase rendered yeast cells ethanol sensitive. Experiments with *sod1* and *sod2* mutants showed that MnSOD but not CuZnSOD is essential for ethanol tolerance. The importance of MnSOD during ethanol stress may be related to its location within the mitochondria, which are the main producers of ROS during respiration (Costa et al., 1993). Moreover, gene expression analysis showed that genes involved in antioxidant defence mechanisms are upregulated in response to ethanol (Alexandre et al., 2001).

2.2 The ethanol stress response

2.2.1 Transcriptional response to ethanol stress

In order to survive and grow under conditions of ethanol stress, yeast acquired a series of stress response mechanisms that depend on a complex network of sensing and signal transduction pathways leading to changes in gene expression profiles and cell metabolic activities (Stanley et al., 2010a). Yeast strains that can easily adapt to different stress conditions and use those signalling pathways in an efficient way are likely to perform well in industrial processes. In the last decades, there have been a number of studies investigating the transcriptional response of *S. cerevisiae* to ethanol stress (Table 1). Some studies compared transcriptomes of parent strains and their ethanol tolerant mutant strains during short-term ethanol exposure (Dinh et al., 2009; Hirasawa et al., 2007; Kasavi et al., 2016; Ma and Liu, 2010a; Ogawa et al., 2000; Stanley et al., 2010b), while other studies directly compared the transcriptional response of *S. cerevisiae* under ethanol stressed and non-stressed conditions (Alexandre et al., 2001; Chandler et al., 2004; Fujita et al., 2006). Moreover, some researchers even followed gene expression profiles during fermentations (Marks et al., 2008; Rossignol et al., 2003; Wu et al., 2006). Although these investigations used different strains, conditions and methods to determine gene expression profiles under ethanol stress, most gene ontology (GO) categories affected by ethanol were comparable. In general, the categories with enhanced gene expression were associated with energy yielding pathways such as glycolysis and TCA-cycle, vacuolar, vesicular and peroxisomal transport, cell

wall organization interactions, lipid metabolism, general stress response, trehalose metabolism, protein destination and ion homeostasis. The GO categories with repressed gene expression on the other hand, are genes involved in energy-demanding growth related processes such as protein synthesis, RNA synthesis and processing, amino acid metabolism and nucleotide metabolism (Stanley et al., 2010a).

Table 1: Gene expression studies related to ethanol tolerance. Adapted from Stanley et al., 2010a; Ma and Liu et al., 2010b.

Strain	Cultivation conditions	Ethanol stress % (v/v)	Source
Parent vs mutant strains			
K701, SR4-3	YPAD, 20°C	10	Ogawa *et al.* (2000)
FY834, K7, S288c	YPD, 30°C	5	Hirasawa *et al.* (2007)
FY834, FY834-A1	YPD, 30°C	10	Dinh *et al.* (2009)
NRRL Y-50316, NRRL Y-50049	YM, 30°C	8	Ma and Liu *et al.* (2010a)
W303-1A, CM1, SM1	YPD, 30°C	6.5	Stanley *et al*. (2010b)
BY4743, BY4743 *pmt7Δ/pmt7Δ,* BY4743 *yhl042wΔ/yhl042wΔ*	YPD, 30°C	8	Kasavi *et al.* (2016)
Stressed vs non-stressed strains			
S288c	YPD, 28°C	7	Alexandre *et al.* (2001)
PMY1-1	YNB, 30°C	5	Chandler *et al.* (2004)
S288c	YPD, 25°C	9	Fuijta *et al.* (2004)
Transcriptome differences during fermentation			
EC1118	Synthetic must, 24°C	Various ethanol concentrations	Rossignol *et al.* (2003)
K701	Sake mash, 15°C	Various ethanol concentrations	Wu *et al.* (2006)
Vin13	Grape juice, 30°C	Various ethanol concentrations	Marks *et al.* (2008)

2.2.2 Functional genomic screens and ethanol stress

In the past decades, there have been numerous reports on functional genomic screens in order to better understand the genetic basis of ethanol tolerance in *S. cerevisiae*. (Table 3). Some research groups used transposon mutagenesis to identify genes required for growth in medium with ethanol versus medium without ethanol (Takahashi et al., 2001), others used the single gene knockout (SGKO) collection to screen for ethanol sensitivity (Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). Unfortunately, there is little overlap in the results between the six disruptome analyses, which may be due to strain and cultivation differences, especially also the ethanol concentrations

used. In general, it appears that mechanisms of ethanol tolerance are dependent on the ethanol concentration used because different genes have been associated with tolerance to 8% (v/v) compared to 11% (v/v) ethanol (Kubota et al., 2004). However, in general, the functional genomic screens for ethanol tolerance report over 200 genes associated with ethanol tolerance and identified different cellular processes affected by ethanol such as intracellular trafficking, including vacuolar protein sorting and vesicular transport, amino acid biosynthesis, transcription, pH homeostasis, peroxisomal transport, aerobic respiration and mitochondrial function (Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). A summarized overview of genes and processes associated with ethanol toxicity is given in Table 2.

Table 2: Genes and processes associated with ethanol toxicity in yeast (adapted from Snoeks et al., 2016).

Cell function and ethanol influence	**Genes**	**References**
Membrane permeabilization		
Cell membrane composition	*OLE1, ERG6,*	van Voorst et al., 2006
Loss of electrochemical gradients and proton-motive force	*PMA1, TRK1*	Lam et al., 2014; Lee et al., 2017
Cytosolic acidification	*VMA and VPS genes*	Duitema et al., 2014;
Protein denaturation		
Trehalose metabolism	*TPS1, TPS2, NTH1, ATH1*	Kim et al., 1996; van Voorst et al., 2006
Heat shock proteins	*HSP12, HSP26, HSP30, HSP78, HSP82, HSP104, SSA3, SSA4, APJ1*	Swinnen et al., 2012; Yoshikawa et al., 2009
DNA damage repair and chromosome integrity	*PAT1, ADE1, KIN3*	Pais et al., 2013; Takahashi et al., 2001
Amino acids and nutrient uptake	*PUT4, PRO1, TAT2, TRP2, TRP5, URA3*	Hirasawa et al., 2007; Swinnen et al., 2015; Takagi et al., 2005
ROS generation	SOD1, SOD2, CTA1, CTT1	Costa, 1997; Ogawa et al., 2000

Moreover, these candidate gene approaches seem limiting in unravelling the complete genetic basis of ethanol tolerance because of its polygenic character. This means that the genetic basis of quantitative traits is a complex interplay between different genes affecting the trait through both direct and interactive effects (Swinnen et al., 2012b). Different research

groups have used quantitative trait loci (QTL) mapping to identify regions in the *S. cerevisiae* genome important for the difference in ethanol tolerance between two divergent strains (Hu et al., 2007; Pais et al., 2013; Swinnen et al., 2012a). Hu and co-workers (2007) identified five QTLs on chromosome 6, 7, 9, 12 and 16, respectively. Swinnen and co-workers (2012b) identified three major QTLs on chromosome 5, 10 and 14, respectively, as well as some additional minor QTLs using pooled-segregant whole-genome sequence analysis to map all QTLs determining high ethanol tolerance between a superior industrial yeast strain and an inferior lab strain. Pais and co-workers (2013) have applied pooled-segregant whole-genome sequence analysis to identify and compare the polygenic basis of maximal ethanol accumulation capacity and ethanol tolerance. They identified eleven and eight QTLs for both traits, respectively, and showed that both traits have a partially different genetic basis. The lack of commonality across these studies, together with strain and medium differences, shows that high ethanol tolerance is a complex trait and can probably be established by combining different causative alleles.

Table 3: Functional genomic screens for ethanol tolerance.

Strain	Cultivation conditions	Ethanol stress % (v/v)	Source
Transposon mutants			
YPH499	YPAD solid, 30°C	6	Takahashi *et al.* (2001)
Single Gene Knockout collection			
BY4741	YPD, solid, 28°C	8, 11	Kubota *et al.* (2004)
BY4743	YPD solid, 30°C	10	Fuijta *et al.* (2006)
BY4742	YPD solid, 30°C	6	van Voorst *et al.* (2006)
BY4742	YPD liquid, 30°C	8	Yoshikawa *et al.* (2009)
BY4741, BY4743	MM4 solid, 30°C	8, 12	Teixeira *et al.* (2009)
Quantitative trait loci mapping			
YH1A and YL1C	YPD liquid, 30°C	16	Hu *et al.* (2007)
VR1-5B and BY4741	YPD solid, 30°C	16, 17	Swinnen *et al.* (2012a)
Seg5 and BY710	YPD liquid, 30°C	18	Pais *et al.* (2013)

2.2.3 Signal transduction pathways involved in ethanol stress

When yeast cells are exposed to environmental stresses, such as ethanol stress, they adapt to the environment through transcriptional, translational and other types of regulation (Gasch et al., 2000). There are two major stress response pathways in *S. cerevisiae*, i.e. the general stress response pathway mediated by two zinc finger transcription factors Msn2 and Msn4 and the heat shock response pathway mediated by the heat shock transcription factor Hsf1 (Gibson et al., 2007). Many genes upregulated by ethanol stress were found to have transcription factor binding motifs for Msn2/4, Yap1 and/or Hsf1 in their upstream sequence (Ma and Liu, 2010a). Ma and Liu (2010a) showed that Msn4 is likely a key regulator of ethanol tolerance, based on its unique transcription profile in an ethanol tolerant yeast. Msn2/4 induce the expression of genes involved in the general stress response pathway via a pentameric cis-acting CCCCT sequence within the promoter region of these genes, the so-called stress-responsive element (STRE). STRE motifs are found in most ethanol-induced

genes. Double deletion mutants of *msn2* and *msn4* show hypersensitivity to different stresses such as heat shock, osmotic stress, ethanol stress and starvation (Moskvina et al., 1998). Moreover, overexpression of *MSN2* under a constitutive promoter increases ethanol tolerance (Watanabe et al., 2009).

Different signalling pathways are involved in the localization of Msn2/4. The primary signalling pathway is the cyclic AMP (cAMP) – protein kinase A (PKA) pathway. The cAMP-PKA pathway regulates yeast proliferation upon nutritional sensing as well as the activity of STRE motifs and heat shock elements (HSE) targeted by Msn2/4 and Hsf1, respectively (Roosen et al., 2005). Other stress responses are regulated by Yap1 and Skn7 (Toone and Jones, 1998). Glucose triggers the G protein-coupled receptor system, Gpr1-Gpa2, to activate adenylate cyclase, Cyr1, to generate high levels of cAMP, which in turn activates PKA and inhibits the general stress response regulated by Msn2/4 (Estruch, 2000). Under stress conditions, cAMP levels and/or PKA activity may be downregulated by inactivation of adenylate cyclase via the Ras proteins. (Thevelein, 1991; Thevelein and de Winde, 1999). When PKA is inactivated, Msn2/4 and Yap1 are rapidly accumulated in the nucleus to trigger the transcriptional stress responses (Toone and Jones, 1998) (Fig. 5). Other pathways involved in the localization of Msn2/4 are the target of rapamycin (TOR) signalling pathway, which transduces stress signals during heat shock, osmotic stress, and nitrogen starvation and the Hog1-MAPK pathway, which is activated during osmotic stress (Gorner et al., 2002; Inoue et al., 1998).

Hsf1 mediates the expression of HSPs that are important for tolerance to various stresses (Pirkkala et al., 2001). Hsf1 binds to heat shock elements (HSE) consisting of short inverted repeats nGAAn in the promoter region of HSP genes. It is known that heat, oxidative stress and ethanol activate Hsf1 in yeast cells (Lee et al., 2000; Sorger and Pelham, 1988). Takemori and colleagues (2006) showed that a *hsf1* mutant yeast strain showed repression of its target genes normally induced during ethanol stress.

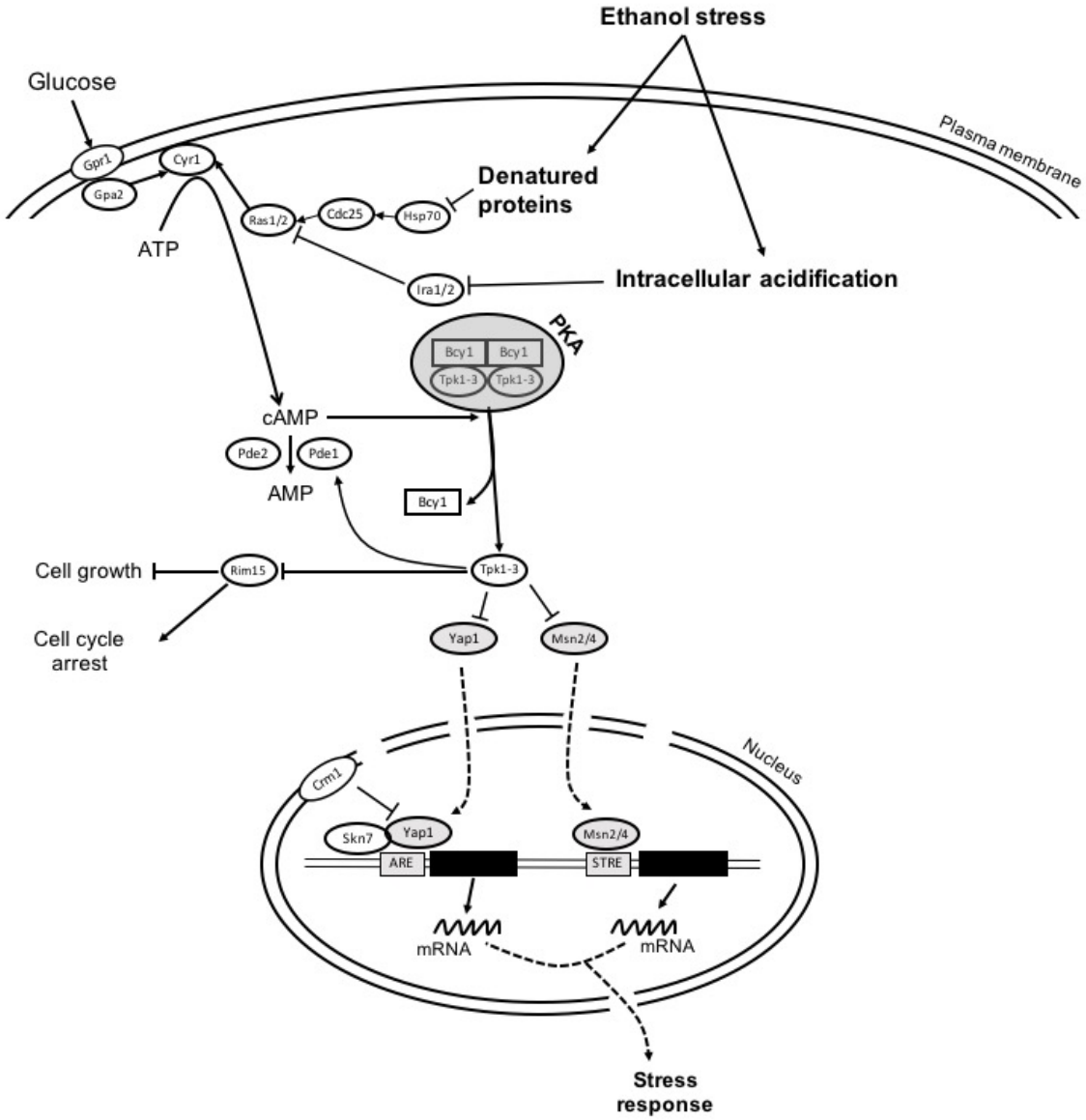


Figure 5: Signal transduction pathways involved in the ethanol stress response in S. cerevisiae, adapted from Ma and Liu (2010b).

2.3 Strain improvement for ethanol tolerance and/or ethanol production

The development of high ethanol production capability in ethanologenic yeast strains has great economic value for the bioethanol production industry (Thammasittirong et al., 2013). To be economically viable, the ethanol titer is usually around 10-14% v/v and the theoretical yield has to be at least 90-93% with respect to the fermentation efficiency for the conversion of glucose into ethanol for first generation bioethanol (Bai et al., 2008). Despite *S. cerevisiae* showing high ethanol tolerance, there have been many strategies employed to improve

ethanol tolerance or production. In this section, we describe the different strategies applied for improving ethanol tolerance and highlight the recent advances.

Research on gene expression analysis in tolerant and non-tolerant strains has led to the identification of target genes involved in ethanol tolerance (Ma and Liu, 2010a; Stanley et al., 2010b). Although it is difficult to alter a complex phenotype like ethanol tolerance by single gene manipulation (deletion or overexpression), several examples have demonstrated successful improvement for this trait (Hirasawa et al., 2007; Krause et al., 2007; Yazawa et al., 2007). Divate and colleagues (2016) have constructed a genetic recombinant *S. cerevisiae* by overexpression of *TPS1* (the gene encoding trehalose-6-phosphate synthase) and deletion of *NTH1* (the gene encoding the trehalose degrading enzyme), that resulted in higher intracellular trehalose accumulation. That strain showed higher ethanol yields compared to the wild type strain when the medium contained more than 15% glucose and increased its tolerance against ethanol from 6 to 10% (v/v). Lam and co-workers (2014) showed that manipulation of ion transport systems by overexpression of the *TRK1* gene, a component of the potassium transport system, and the H^+-ATPase gene, *PMA1*, in S288c increased ethanol production by around 30% compared to the wild type strain and by 10% compared to industrial strains.

However, simultaneous manipulation of multiple genes is more desirable for influencing complex traits, which is difficult to achieve with the classical metabolic engineering strategies. With the global transcription machinery engineering (gTME) technique, reprogramming of the transcriptome was achieved in order to obtain mutant strains with improved ethanol tolerance (Alper et al., 2006). The underlying principle involves creating mutant libraries of transcription factors that control a large set of genes, followed by screening for the mutants with the desired phenotype (Lanza and Alper, 2011). Mutagenesis of transcription factor (TF) Spt15 allowed selection of the SPT15-300 mutant strain derived from BY4741, which was found to have a triple mutation in Spt15 (Phe177Ser, Tyr195His and Lys218Arg). Spt15 is a TATA-binding protein, which is a component of the RNA polymerase II transcription factor D (TFIID) complex. This engineered strain showed increased tolerance to high glucose and ethanol concentrations, as well as improved ethanol production (Alper et al., 2006). Yang and co-workers (2011) applied the same method to create strains with enhanced ethanol

tolerance by generating mutations in *SPT15* and compared the global transcriptional profiles of two selected ethanol tolerant strains with that of the control, which identified several genes associated with ethanol tolerance. Recently, Tan and colleagues (2016) could improve the ethanol tolerance of *Zymomonas mobilis* by applying gTME by random mutagenesis of the global transcription factor RpoD protein.

Another way to obtain improved strains is adaptive evolution. Adaptive evolution can be a successful strategy to improve yeast strains for ethanol tolerance as well as maximal accumulation. It is known that yeast and bacteria induce random mutations in the genome under environmental stress, leading to survival of fitter strains (Galhardo et al., 2007). Several research groups have selected ethanol tolerant mutants producing higher concentrations of ethanol using adaptive evolution over multiple generations (Cakar et al., 2005; Chen and Xu, 2014; Dinh et al., 2008; Stanley et al., 2010c). Unfortunately, the mechanisms and genetic changes underlying this adaptation have mostly remained unexplored. On the other hand, Voordeckers and co-workers (2015) made a comprehensive analysis of the molecular mechanisms involved in adaptation to high levels of ethanol. In their study, whole-genome sequence analysis of different evolved populations and adapted clones was performed after long-term experimental evolution and revealed that many mutations target functional modules involved in stress response, cell cycle regulation, DNA repair and respiration.

Another possibility to improve yeast strains for complex traits without the need for insight into the molecular mechanisms is the powerful technology of “genome shuffling”. Yeast has a short life cycle that makes it possible to rapidly generate many consecutive rounds of multi-parental crosses in a short time (Steensels et al., 2014). Genome shuffling aims to combine many different beneficial mutations in the same cell, leading to additive or synergistic effects on the trait of interest. One way to obtain this, is by generating a genetically diverse starting population by mutagenesis using chemicals or UV radiation, followed by screening or selection of the best-performing strains. Thereafter, the genomes of these strains are shuffled by protoplast fusion or sporulation and mating in order to create novel combinations of beneficial mutations (Snoek et al., 2015). In the last decade, researchers have applied this method to improve ethanol tolerance and/or ethanol accumulation capacity of yeast strains. (Hou, 2010; Shi et al., 2009; Snoek et al., 2015; Zheng et al., 2013). Other research groups

have combined metabolic engineering with genome shuffling in order to improve the performance of yeast strains in bioethanol production (Demeke et al., 2013a; Jingping et al., 2012; Tao et al., 2012; Wang and Hou, 2010; Wang et al., 2012).

CHAPTER II

Identification of causative alleles involved in high ethanol tolerance

1. Introduction

Ethanol tolerance is an important characteristic of the yeast *S. cerevisiae.* Because yeast is a superb ethanol producer, many industrial applications, such as the production of alcoholic beverages and biofuel production, rely on this micro-organism (Snoek *et al.*, 2016). However, *S. cerevisiae* is also sensitive to high ethanol concentrations especially under very high gravity conditions (Ma and Liu, 2010). Ethanol interferes with the plasma membrane lipid organization, leading to the dissipation of the transmembrane electrochemical potential, inhibits membrane transporters and crucial glycolytic enzymes and induces the generation of ROS (Casey and Ingledew, 1986; Van Uden, 1985). Throughout the years, significant efforts have been made to study the ethanol stress response (Ding *et al.*, 2009; Snoek *et al.*, 2016; Stanley *et al.*, 2010). This has contributed to increased understanding of the molecular processes underlying ethanol toxicity in *S. cerevisiae*. However, ethanol tolerance has been mostly studied utilizing laboratory strains, such as S288c and their derivatives, with low to moderately high ethanol concentrations (5-12%), providing little information about the genetic elements that lead to the high ethanol tolerance of natural and industrial yeast strains.

Throughout the last two decades, there have been several linkage mapping experiments reported in order to identify quantitative trait loci (QTL) of complex traits in yeast (Liti and Louis, 2012). Most traits of industrial importance are polygenic, therefore complex and difficult to study. Due to the advent of the genomic revolution and high-throughput sequencing technologies, large numbers of single-nucleotide polymorphisms (SNPs) can be used as natural markers instead of scoring genetic markers by hybridization of genomic DNA to gene expression micro-arrays (Cubillos, 2016; Steinmetz *et al.*, 2002). The latest quantitative genetic studies in yeast have uncovered genetic variants for many traits involved in fermentation such as nitrogen consumption (Brice *et al.*, 2014); sulphite resistance (Zimmer *et al.*, 2014); aroma production (Steyer *et al.*, 2012); fermentation kinetics (Kessi-Perez *et al.*, 2016); low glycerol/high ethanol production (Hubmann *et al.*, 2013); thermotolerance (Yang *et al.*, 2013) and ethanol tolerance and production (Pais *et al.*, 2013; Swinnen *et al.*, 2012) that could be useful in multiple applications of industrial processes.

Swinnen and co-workers (2012) applied pooled-segregant whole genome sequencing analysis for QTL mapping of high ethanol tolerance in a former Brazilian bioethanol production strain VR1 in order to identify causative mutations underlying high ethanol tolerance. For this approach, VR1-5B, a haploid segregant of VR1 was crossed with the laboratory strain BY4741. The diploid hybrid was sporulated and segregants were scored for ethanol tolerance by scoring their growth on YP medium containing different ethanol concentrations. Two pools were constructed by classifying 136 segregants tolerant to at least 16% ethanol in a first pool, while the second pool contained 31 segregants from the first pool that were tolerant to at least 17% ethanol. Genomic DNA from both pools and parent strains were extracted and sequenced by Illumina sequencing. The sequences of both pools were aligned to the reference S288c genome sequence and the SNP variant frequencies of both pools plotted against the chromosomal position. Swinnen and co-workers (2012) identified three major QTLs located on chromosome V (QTL1), X (QTL2) and XIV (QTL3) and additional minor QTLs appeared for the second pool, which was selected under more stringent conditions. The three loci were validated by statistical analysis of the segregation of distinct SNPs in the individual selected segregants. The causative genes of the two loci with the strongest linkage were identified using reciprocal hemizygosity analysis. The *URA3* gene of the superior parent was identified as the causative allele in QTL1. While in QTL3, three closely located alleles ($MKT1_{VR1\text{-}5B}$, $SWS2_{BY4741}$ and $APJ1_{VR1\text{-}5B}$) were identified as the causative alleles for high ethanol tolerance.

In this chapter, we characterize the relevance of the causative alleles identified for high ethanol tolerance in previous research of Swinnen and co-workers (2012). In addition, we describe the identification of a new causative allele involved in high ethanol tolerance using fine-mapping, sequence analysis and reciprocal hemizygosity analysis. Further, we investigate the contribution of the different superior alleles to improve ethanol tolerance in the inferior background.

2. Results

2.1. Auxotrophic mutations reduce tolerance to high ethanol concentrations of *Saccharomyces cerevisiae*

2.1.1. Growth specificity of auxotrophic mutations to high ethanol concentrations and other stresses

Analysis of genetic elements important for very high ethanol tolerance of VR1 by linkage mapping with a laboratory strain revealed that the *ura3Δ0* mutation of the laboratory strain is causative for low ethanol tolerance. Furthermore, other auxotrophic mutations also reduce tolerance to high ethanol concentrations although to different extents (Swinnen *et al.*, 2015).

A number of studies screened single gene knockout (SGKO) collections for ethanol sensitivity to moderate ethanol concentrations (6 - 12%), however none of those studies associated auxotrophic mutations with ethanol tolerance. To evaluate whether the association of auxotrophic mutations to ethanol tolerance is concentration dependent, we compared the growth of the auxotrophic BY4741 and BY4742 strains with that of their prototrophic S288c counterparts on YP medium containing moderate to high concentrations of ethanol (10 – 16%) (Fig. 6A). Interestingly, the auxotrophic strains grew faster than the prototrophic strains on 10% and 12% ethanol, whereas the opposite was observed with 14% and 16% ethanol (Fig. 6A). When 2% glucose was added to the medium, no difference in growth in the presence of 10% ethanol was observed, whereas at higher concentrations, the prototrophic strains again grew better than the auxotrophic strains (Fig. 6B).

The better growth of the prototrophic strains in the presence of high ethanol concentrations may be due to an inhibition of uptake of auxotrophic nutrients, but the better growth of the auxotrophic strains at moderate ethanol concentrations is more difficult to explain. Prototrophic strains must have a balance between uptake and biosynthesis of the nutrients, whereas auxotrophic strains can only take up available nutrients from the medium. Both systems, transport and biosynthesis, require energy, and the presence of ethanol may affect

the balance toward a more unfavourable situation in the prototrophic strains. BY4741 and BY4742 are derivatives of S288c. These strains are in principle isogenic except for the auxotrophic markers and unknown background mutations. BY4741 and BY4742 have the same auxotrophies for *his3Δ1*, *leu2Δ0*, and *ura3Δ0*, while they vary for *met15Δ0* and *lys2Δ0*, respectively. Hence, the small differences in ethanol tolerance between BY4741 and BY4742 may be due to this difference in auxotrophies or background mutations which may make the implicated nutrients growth-limiting under ethanol conditions (Ding et al., 2013; Hanscho et al., 2012).

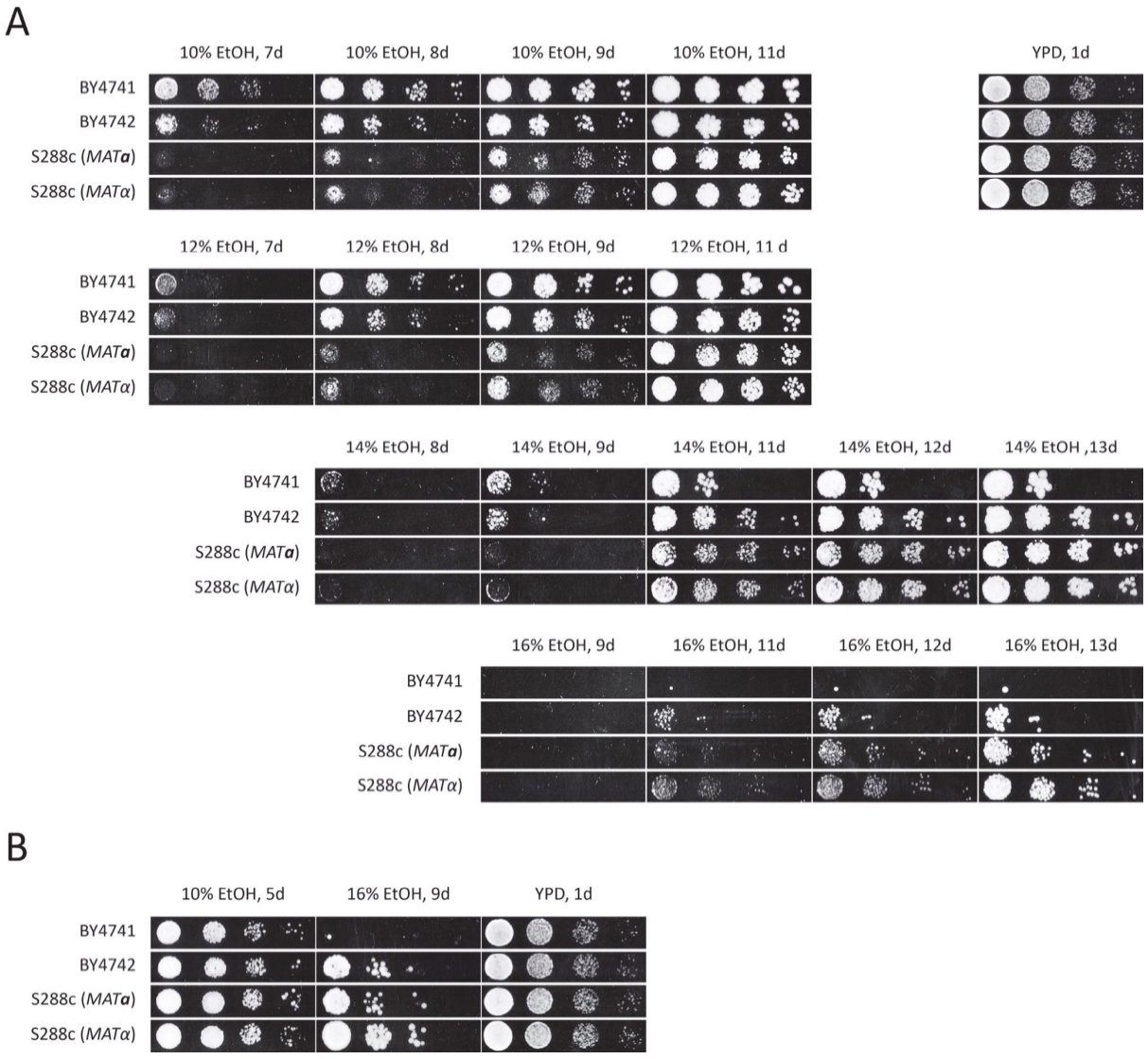


*Figure 6: Growth of 10-fold dilution range of the laboratory BY4741 (MAT**a**) and BY4742 (Matα) strains and their prototrophic counterparts (S288c MAT**a** and S288c Matα) on YP media containing 10 – 16% (A) and 10% and 16% ethanol (B). The growth was tested at 30°C and at different periods of time, as indicated.*

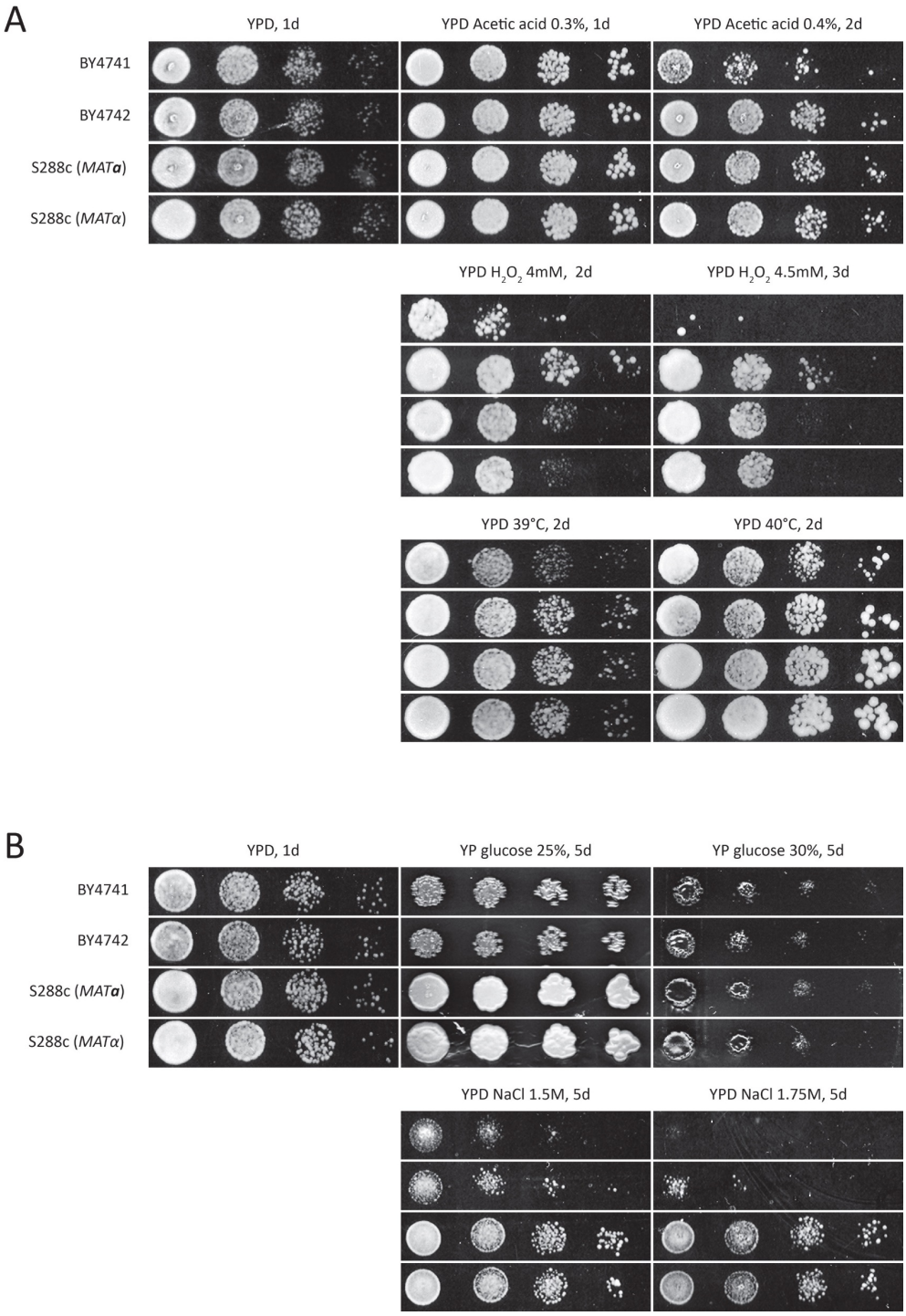


*Figure 7: Growth of 10-fold dilution range of the laboratory BY4741 (MAT**a**) and BY4742 (MATα) strains and their prototrophic counterparts (S288c MAT**a** and S288c MATα) on YPD medium with different stress factors; (A) 0.3% or 0.4% (v/v) acetic acid at pH 5 and 4.0 mM or 4.5 mM hydrogen peroxide. The heat tolerance was investigated at 39°C and 40°C; (B) 25% and 30% glucose and 1.50 M or 1.75 M NaCl. The growth was tested at 30°C except for heat tolerance (see temperature indicated) and at different periods of time, as indicated.*

In addition, we determined tolerance to other stress factors on YPD medium: acetic acid at pH 5, hydrogen peroxide, high temperature, and osmotic stress. The doses that were applied are generally considered to cause high levels of stress and, under our conditions, they also caused a strong growth delay on plates (Fig. 7). No significant differences between the auxotrophic and prototrophic strains were observed for acetic acid, hydrogen peroxide and high temperature stress. Nevertheless, under all conditions, BY4741 was somewhat more sensitive than BY4742 and both S288c strains (Fig. 7A). On the other hand, under conditions of osmotic stress caused by high glucose or NaCl concentrations, the prototrophic strains grew significantly better than the auxotrophic strains (Fig. 7B). Thus, under conditions of osmotic stress, auxotrophic and prototrophic strains grew differently, which was similar to that under conditions of high ethanol stress.

2.1.2. Effect of high ethanol concentrations on nutrient uptake in *S. cerevisiae*

In order to determine whether the growth inhibition of auxotrophic strains under stress is due to an impairment of nutrient uptake, we compared the growth of the prototrophic S288c strains with that of the S288c *ura3-52Δ* and S288c *leu2Δ* strains on minimal medium with limiting amounts of uracil and leucine, respectively, combined with different stress factors (high ethanol and NaCl concentrations and also high temperature, as a control).

First, we determined the minimal amounts of uracil and leucine that are required for growth of those auxotrophic strains by performing a spot assay on minimal medium with different concentrations of the auxotrophic nutrient. The minimal auxotrophic nutrient concentration was defined as the concentration that had to be supplemented to the medium at which growth was still visually the same as growth on the control plate containing an excess of the auxotrophic nutrient concentration. The S288c *ura3-52Δ* strain was not able to grow on minimal medium containing less than 0.05 mM uracil (Fig. 8A), while the S288c *leu2Δ* strain was not able to grow on minimal medium containing less than 0.5 mM leucine (Fig. 8B). In the case of the S288c *ura3-52Δ* strain, growth on minimal medium containing 0.05 mM uracil was inhibited by high osmotic stress (Fig. 9A), high temperature (Fig. 9B), and high ethanol concentrations (Fig. 9C). Hence, thermo-tolerance was also reduced, as opposed to what was previously observed for rich medium (Fig. 7A). On the other hand, no effect on growth of the

S288c *leu2Δ* strain on minimal medium containing 0.5 mM leucine was observed under all the conditions mentioned above (Fig. 10).

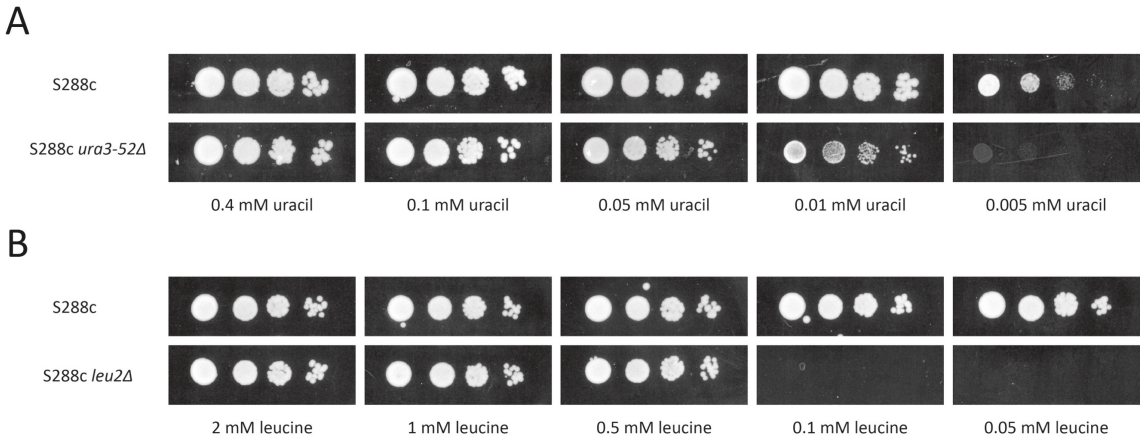


Figure 8: Growth of prototrophic strain S288c and S288c ura3-52Δ and S288c leu2Δ auxotrophic strains on medium containing different concentrations of the auxotrophic nutrient. (A) In order to determine the minimal concentration of uracil that is required to allow growth of the S288c ura3-52Δ strain on minimal medium, a 10-fold dilution range of the strain was spotted onto CSM-URA with different concentrations of uracil. (B) In order to determine the minimal concentration of leucine that is required to allow growth of the S288c leu2Δ strain on minimal medium, a 10-fold dilution range of the strain was spotted onto CSM-LEU with different concentrations of leucine. The cells were incubated for 2 days at 30°C.

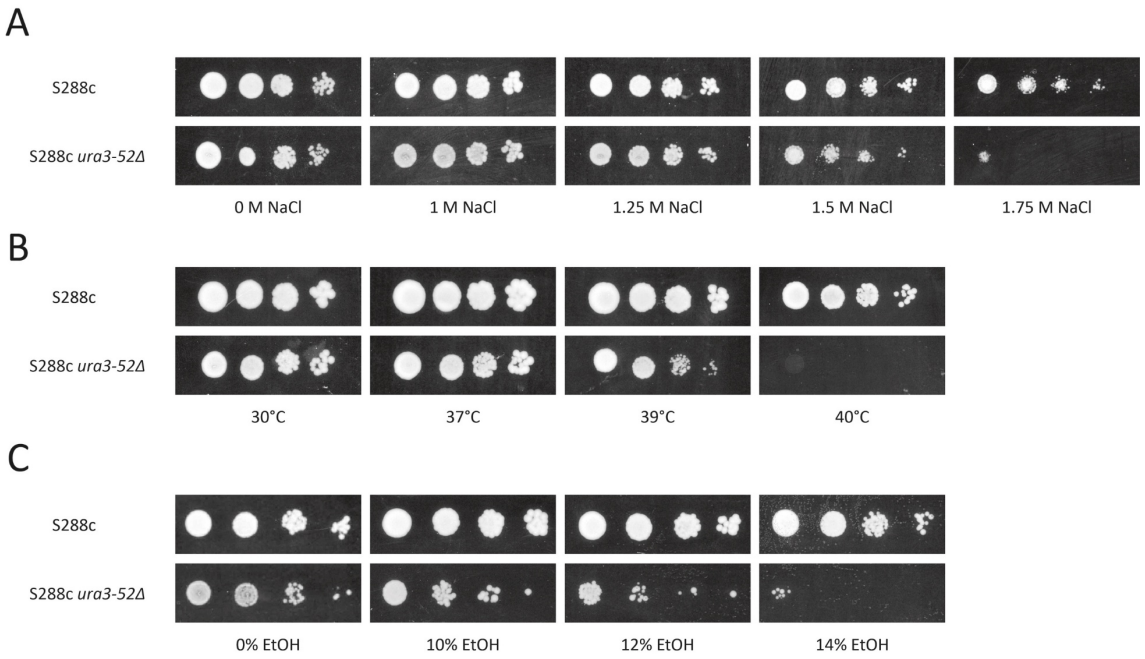


Figure 9: Growth of a 10-fold dilution range of prototrophic strain S288c and the auxotrophic S288c ura3-52Δ strain on minimal medium containing 0.05 mM uracil tested in combination with different concentrations of NaCl for 6 days at 30°C (A); at different temperatures for 3 days (B); and at different concentrations of ethanol (% v/v) for 7 days at 30°C (C).

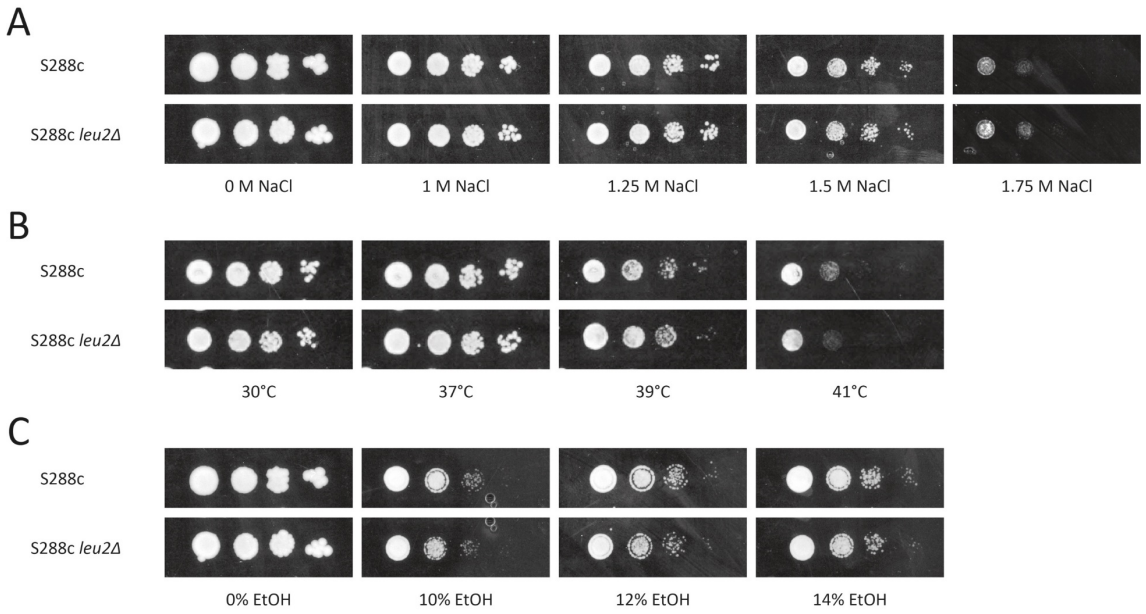


Figure 10: Growth of a 10-fold dilution range of prototrophic S288c strain and auxotrophic S288c leu2Δ strain on minimal medium containing 0.5 mM leucine tested in combination with different concentrations of NaCl for 5 days at 30°C (A); at different temperatures for 2 days (B); and at different concentrations of ethanol (% v/v) for 7 days at 30°C (C).

Furthermore, we investigated the effect of different ethanol concentrations on the rate of uptake of uracil and leucine. In both cases, the uptake was inhibited in the presence of high ethanol concentrations. Moreover, the rate of transport of uracil was less sensitive to high ethanol concentrations than the rate of transport of leucine. The rate of uptake of radiolabelled uracil decreased 40% in the presence of 18% (v/v) ethanol, while the rate of uptake of leucine decreased 80% under the same conditions (Fig. 11). On the other hand, the absolute rate of transport of uracil was 500-fold lower than that of leucine, already under unstressed conditions, which can explain why strains with uracil auxotrophy are more sensitive to high ethanol stress than strains with leucine auxotrophy. The reason for the increase in the rate of uracil uptake with moderate ethanol concentrations is not clear. It could be due to a direct effect of ethanol on the protein structure of the uracil transporter or to an indirect effect of ethanol on the structure of the phospholipid membrane.

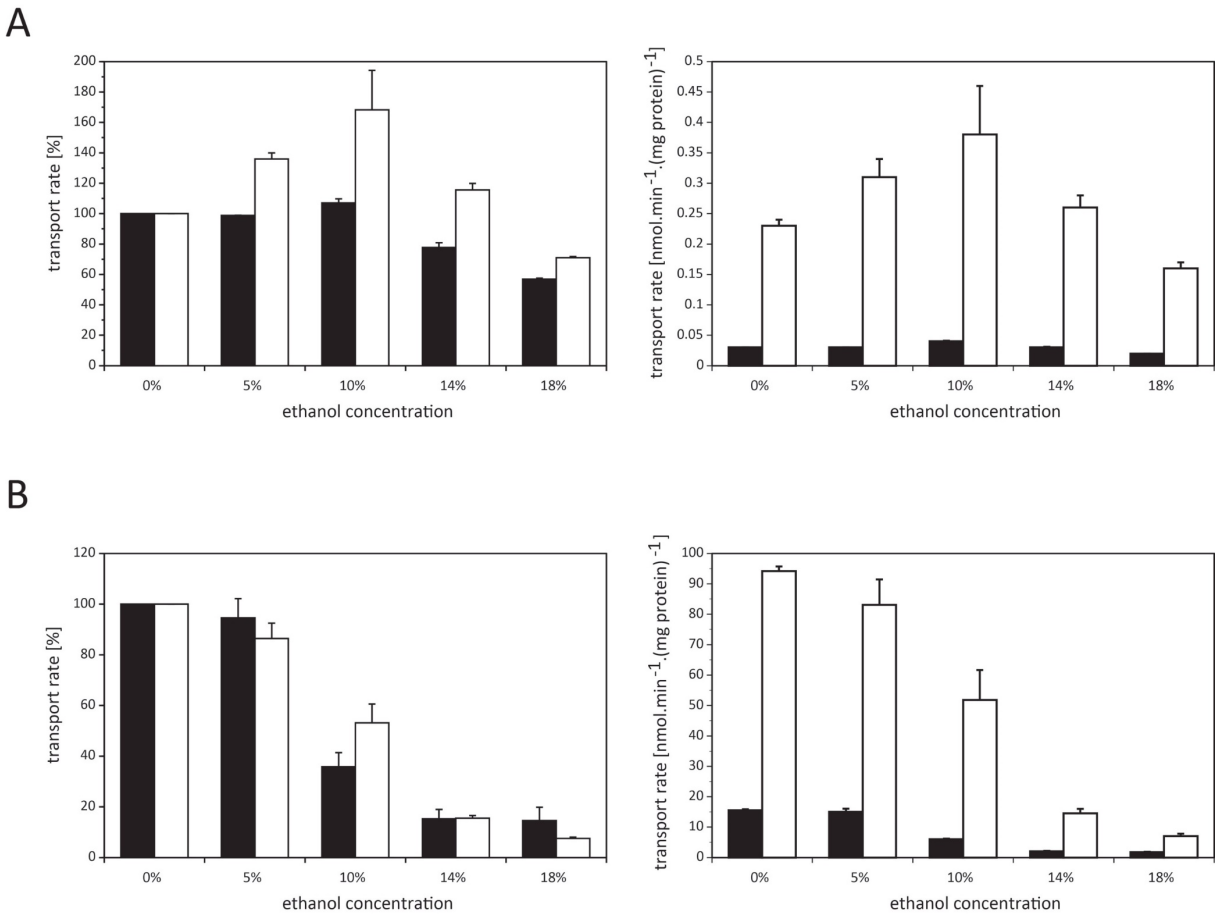


Figure 11: Uptake of uracil and leucine in prototrophic and auxotrophic strains in the presence of different ethanol concentrations (% v/v). Shown are rates of uptake of 0.05 mM uracil by the S288c (black bars) and S288c ura3-52Δ (white bars) strains (A) and rates of uptake of 2.5 mM leucine by the S288c (black bars) and S288c leu2Δ (white bars) strains (B). The transport activity measured in the presence of different concentrations of ethanol is expressed as a percentage of transport in the absence of ethanol (left) and in nmol per minute per milligram of protein (right). Error bars represent the standard deviation from three biological replicates.

2.2. Identification of the causative gene for high ethanol tolerance in QTL2 on chromosome X

2.2.1. Fine-mapping of QTL2 and sequence analysis

The QTL mapping experiment of Swinnen and co-workers (2012) revealed a significant linkage of an approximately 58 kb locus on chromosome X (QTL2). This QTL showed a weaker link, with only 72,9% of the segregants showing VR1-5B inheritance compared to the stronger QTL1 and QTL3. Fine-mapping of the region by PCR-based scoring of the SNP markers in the individual segregants allowed us to confirm the area with the strongest link. As shown in figure 12, calculations of the P values revealed the strongest link between the SNP markers S4 - S7, which defined a smaller locus of approximately 29 kb within QTL2. This region contained 16 genes and hence enabled us to prioritize our further work. The nucleotide polymorphisms between VR1-5B and BY4741 were verified by Sanger sequencing. We observed five genes with polymorphisms in their open reading frame (ORF) and putative regulatory sequences, eight genes with only polymorphisms in their regulatory sequences and three genes with no mutations (Fig. 12).

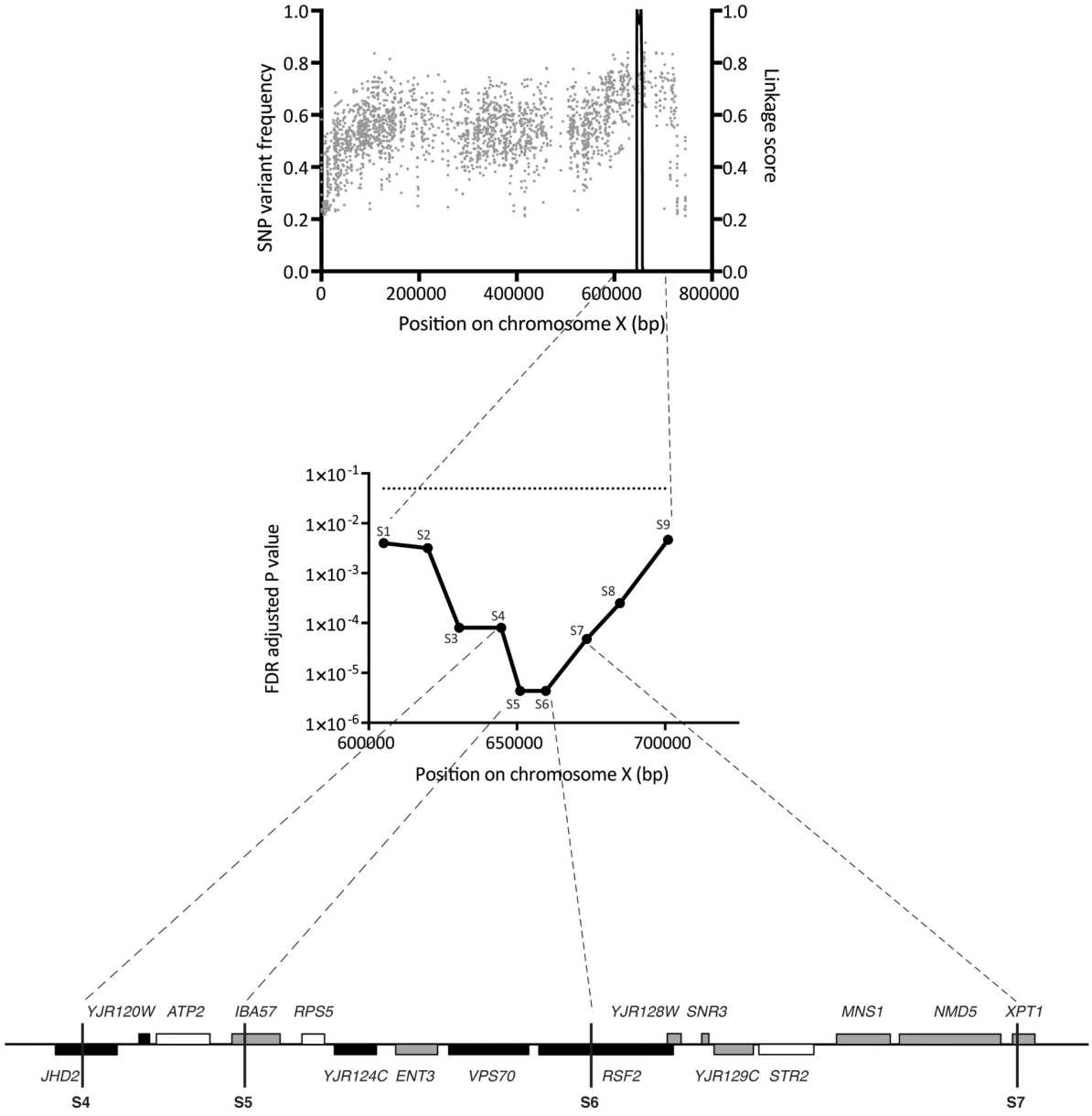


Figure 12: Fine-mapping of QTL2 on chromosome X. Upper plot shows the region corresponding to QTL2 of which linkage to the phenotype of interest was confirmed by scoring selected marker sites in individual segregants. Scored marker sites are indicated (S4-S7). For each marker site, the p-value indicates the probability to be linked to the phenotype by chance according to a binomial distribution (middle plot). Lower plot: zoom in on the genes in the experimentally confirmed region corresponding to QTL2 (29 kb). Black bars: genes with non-synonymous mutations in the coding region; grey bars: genes with mutations in the promotor or terminator; white bars: genes without mutations.

2.2.2. Functional analysis of candidate genes reveals one causative gene for QTL2

After sequence analysis of the fine-mapped locus, genes carrying non-synonymous mutations in their coding region were first selected as candidate causative genes for functional analysis. True causative genes in QTL2 were identified using reciprocal hemizygosity analysis (RHA) (Steinmetz *et al.*, 2002). For each candidate causative gene, we constructed a set of two heterozygous strains by crossing the parental strains, either containing or lacking the candidate gene. As a result, each diploid strain had a different allele of the candidate gene while the other copy of the gene was deleted, i.e. they carried either the VR1-5B- or BY4741-derived allele from the candidate gene (Fig. 13). Phenotypic analysis on YPD plates with 16% (v/v) ethanol showed a clear difference in ethanol tolerance between the two heterozygous strains carrying a different allele of *VPS70* (Fig. 13). The strain with the VR1-5B-derived allele grew very well in the presence of 16% (v/v) ethanol, whereas the strain with the BY4741-derived allele showed reduced growth in the presence of 14% (v/v) ethanol and almost no growth in the presence of 16% (v/v) ethanol (Fig. 13), indicating that *VPS70* carries a causative mutation responsible for the link of QTL2 with high ethanol tolerance. Except for a putative role in sorting of vacuolar carboxypeptidase Y to the vacuole (Bonangelino *et al.*, 2002), no link with ethanol tolerance for *VPS70* has been reported previously. This may be due to the fact that all previous analyses of yeast ethanol tolerance were performed with laboratory strains and with much lower ethanol concentrations although other members of the *VPS* family have been previously linked to ethanol tolerance (Ma and Liu, 2010).

Sequence analysis of *VPS70* in both parental backgrounds (VR1-5B and BY4741) revealed eleven SNPs in the ORF, changing four amino acids in the sequence of the gene product, and no SNPs were detected in the putative promotor and terminator regions (Fig. 14A). Sequence analysis of *VPS70* in the superior diploid parent VR1 demonstrated that VR1 is heterozygous for the *VPS70* allele and that the other allele of *VPS70* contains an extra amino acid change in the protein sequence (Fig. 14A). Interestingly, Voordeckers and co-workers (2015) found also a mutation $VPS70^{C595A}$ during a long-term evolutionary adaptation experiment with increasing levels of ethanol which conferred a fitness advantage in the high ethanol environment. The mutation (C595A) in the gene *VPS70* alters the same amino acid as the mutation present in our superior parent VR1-5B ($VPS70^{C596T}$). The fact that two independent research lineages

found a mutation that alters the same amino acid suggests the potential of this finding for targeted improvement of ethanol tolerance in other yeast strains.

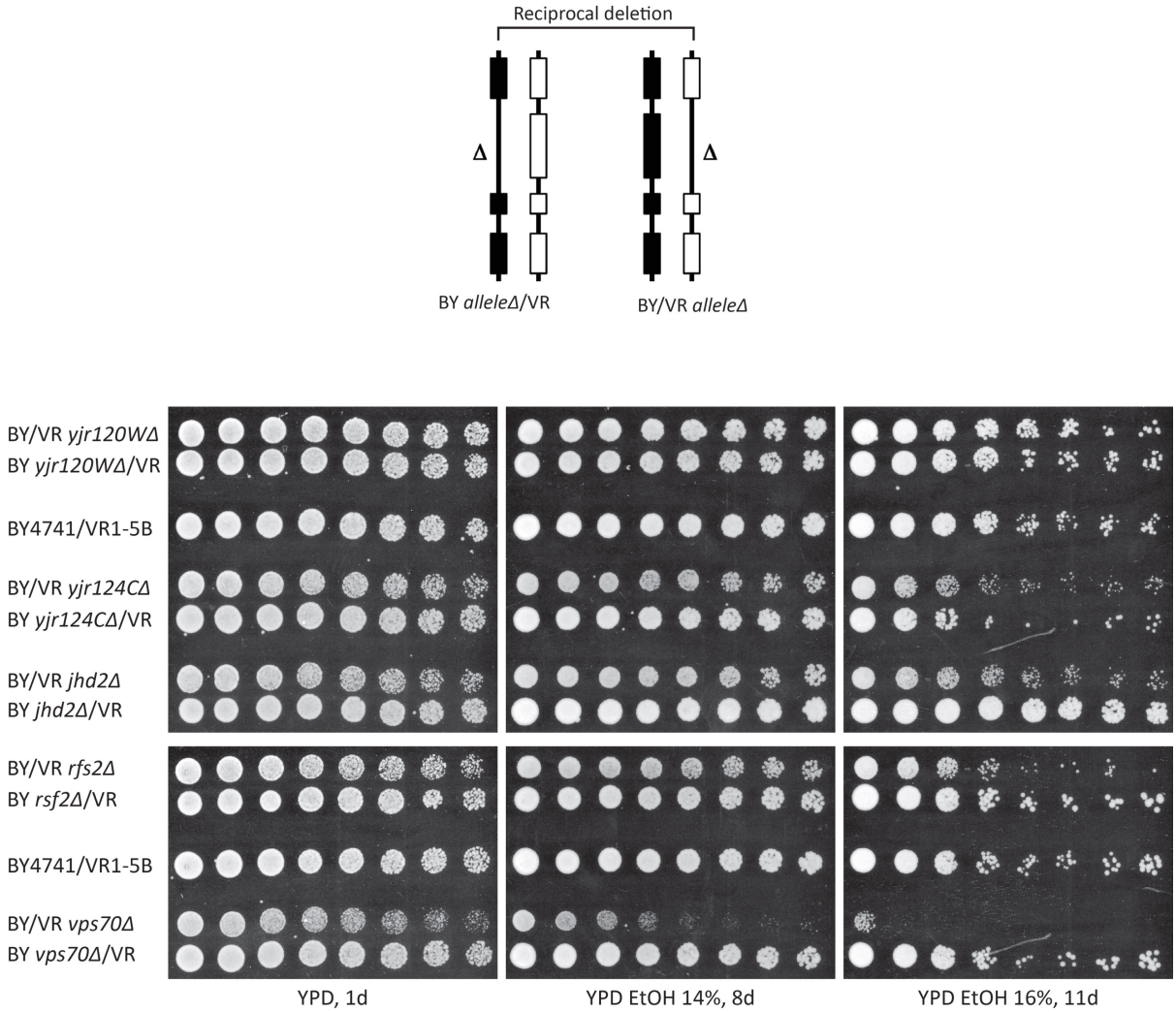


Figure 13: Reciprocal hemizygosity analysis for the genes with non-synonymous mutations in the coding region located in the fine-mapped region. Two heterozygous strains were constructed by crossing the original superior parent VR1-5B with the inferior parent BY4741, carrying a deletion in the candidate causative allele. This resulted in two different diploid strains, each with one functional allele of the candidate gene, originating from either the superior or inferior parent. Ethanol tolerance of the two heterozygous strains was compared using a dilution spot assay on a YPD plate with 14% - 16% (v/v) ethanol and a YPD plate without ethanol as control.

In order to exclude a loss-of-function allele for *VPS70* in one of the parental backgrounds, we determined the deletion phenotypes of *VPS70* in VR1-5B and BY4741 (Fig. 14B). In both the haploid parental backgrounds, *vps70Δ* strains grew worse in the presence of high ethanol concentrations compared to the wild type strains. In the VR1-5B background, deletion of *VPS70* resulted in reduced growth on 16% (v/v) ethanol, while growth on lower concentrations of ethanol did not show any difference between wild type and deletion strain. Likewise, in the BY4741 background, the deletion of *VPS70* resulted in reduced growth in the presence of 14% (v/v) ethanol and equal growth in the presence of lower ethanol concentrations. These results indicate that there is no loss-of-function allele in both parental backgrounds and that *VPS70* is only important for high ethanol tolerance. The fact that there is no difference in ethanol tolerance between the wildtype and *vps70Δ* strain in the presence of low ethanol concentrations might be the reason why *VPS70* has never been identified in screens for ethanol sensitivity with the single-gene knockout collection.

A

		ORF *VPS70*				
	position	**9**	**147**	**199**	**327**	**708**
	S288c	K	E	P	R	G
VR1 tetrad	VR1 (A)	E	G	L	K	D
	VR1 (B)	E	E	L	K	D
	VR1 (C)	E	E	L	K	D
	VR1 (D)	E	G	L	K	D
	VR1-5B	E	E	L	K	D

B

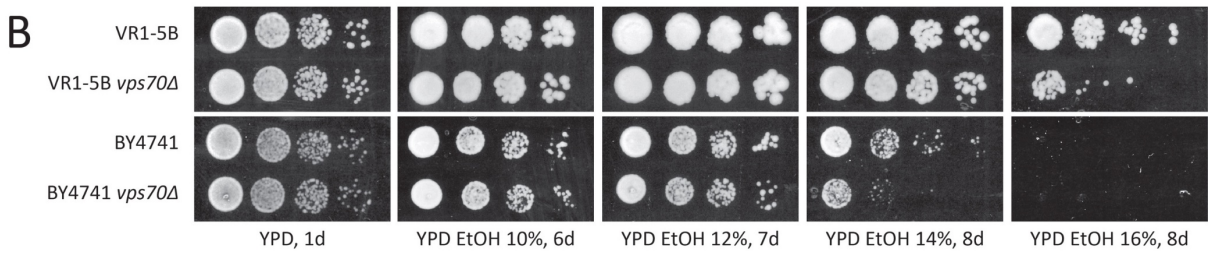


Figure 14: (A) Representation of amino acid changes in ORF VPS70 in a complete tetrad of superior parent VR1 and the haploid segregant VR1-5B compared to S288c. (B) Ethanol tolerance of vps70Δ strains in the haploid parental background. The ethanol tolerance of the vps70 deletion strains and wild type strains was determined by scoring growth of 10-fold dilutions on YPD plates with different concentrations of ethanol (% v/v) after the indicated time points.

2.3. Further research on the superior allele *APJ1* for high ethanol tolerance

Swinnen and co-workers (2012) identified three closely located genes in chromosome XIV (QTL3) affecting high ethanol tolerance: *MKT1*, *SWS2* and *APJ1*. In this paragraph, we discuss the contribution of *APJ1* to high ethanol tolerance. *APJ1* was identified as a causative gene by reciprocal hemizygosity analysis. The presence of the VR1-5B allele of the *APJ1* gene resulted in higher ethanol tolerance in the heterozygous background compared to presence of the BY4741 allele (Fig. 15A). In addition, Swinnen and co-workers (2012) showed that in the BY4741 background, the *apj1Δ* strain grew clearly better in the presence of higher ethanol concentrations than the wild type strain. Deletion of *APJ1* in the VR1-5B background resulted in a small improvement in ethanol tolerance compared to the wild type strain (Fig. 15B). Together, these results suggested that the *APJ1* gene product acts negatively on ethanol tolerance, at least under the test conditions applied. Sequence analysis revealed no non-synonymous mutations in the ORF but revealed polymorphisms in the putative promotor and terminator region (Fig. 16). This suggested that a difference in expression level of the two parental alleles might account for the difference in ethanol tolerance.

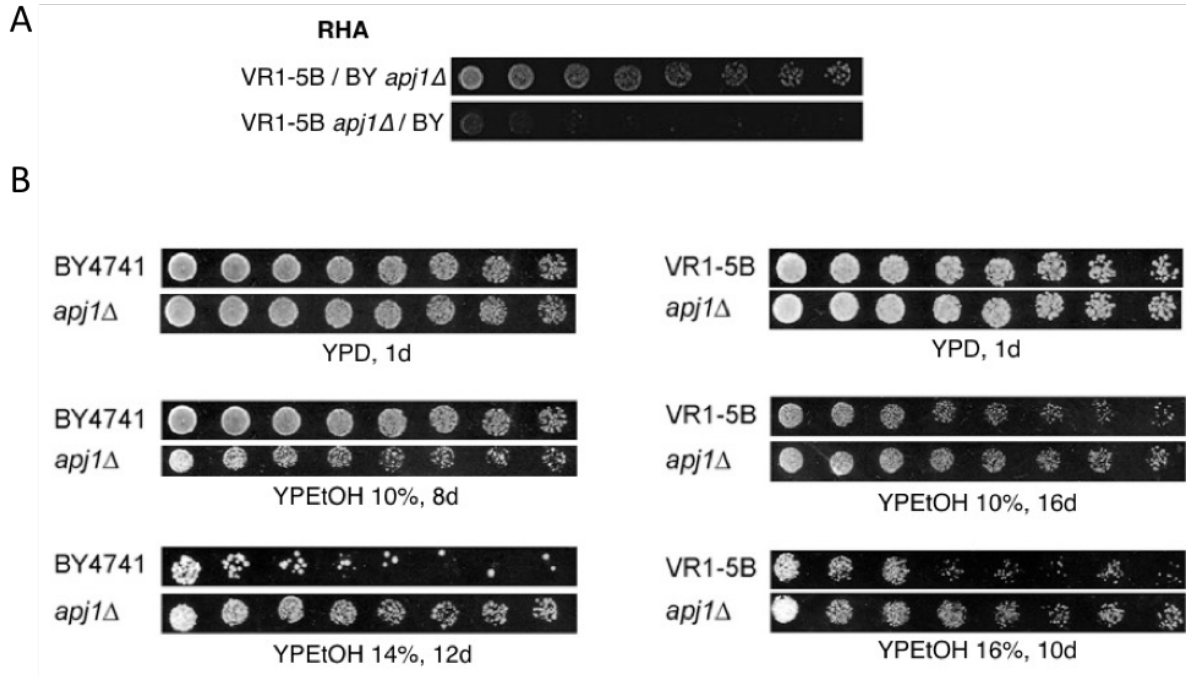


Figure 15: Effect of APJ1 on ethanol tolerance. (A) Reciprocal hemizygosity analysis of the candidate gene APJ1 in the VR1-5B/BY4741 hybrid background carrying either the VR1-5B allele or the BY4741 allele. Ethanol tolerance of the heterozygous strains was determined by scoring growth of the twofold dilutions on 16% (v/v) ethanol after 9d. (B) The ethanol tolerance of BY4741 (inferior parent) and VR1-5B (superior parent) and the apj1Δ mutants thereof were determined by scoring growth of twofold dilutions in the presence of different ethanol concentrations (10%-16% (v/v) ethanol) at the indicated time points.

Therefore, we measured the expression of *APJ1* in the BY4741 and VR1-5B strains during fermentation by Real-time PCR. The results revealed a higher expression level of *APJ1* in the BY4741 strain (normalized to 1.0 ± 0.19) compared to the VR1-5B strain (0.43 ± 0.12) especially at the beginning of the fermentation (Fig. 16). The expression of *APJ1* is approximately 2.5-fold higher in BY4741 compared to VR1-5B. Although the expression in VR1-5B was lower, it was clearly detectable, consistent with deletion of *APJ1* causing a further slight improvement in ethanol tolerance of VR1-5B. These results confirm that the difference in expression level of *APJ1* at the beginning of the fermentation may account for the difference in ethanol tolerance between the two parental strains, although we cannot rule out other possibilities due to differences in experimental conditions between the growth assays and the fermentations.

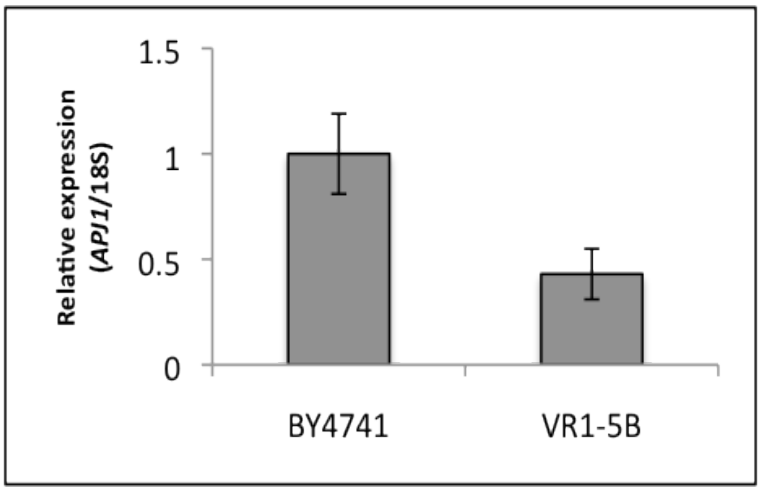
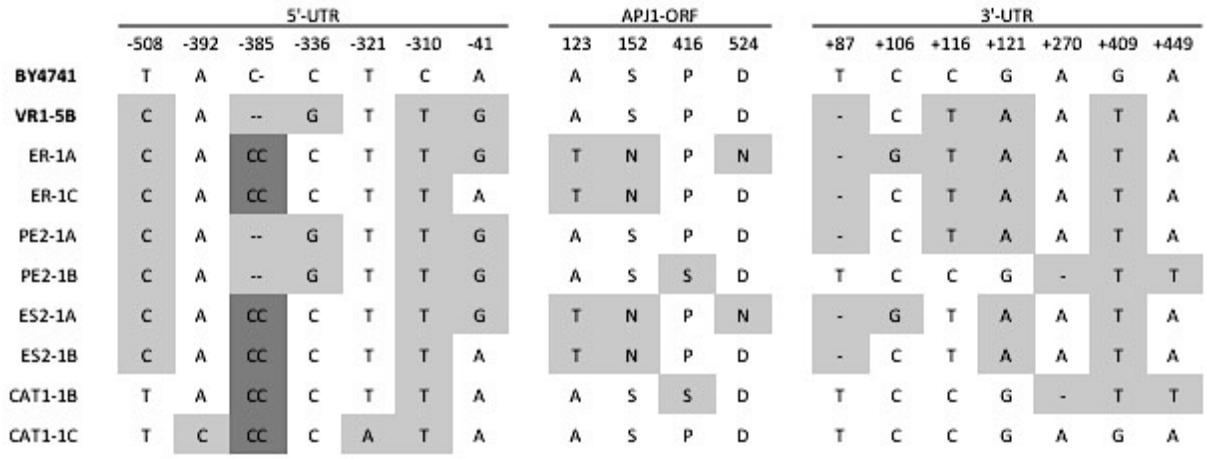


Figure 16: Real-time PCR determination of the expression of APJ1 in the BY4741 and VR1-5B strains at the beginning of the fermentation. Samples from 3 independent cultures were taken after 4h of fermentation. The normalization was done using 18S as house-keeping gene.

2.3.1. Evaluation of the possible improvement of ethanol tolerance in industrial yeast strains by *APJ1* deletion.

Because the deletion of the *APJ1* allele seemed to improve ethanol tolerance of the parental strains to some extent, we might be able to improve other industrial yeast strains for ethanol tolerance by deleting *APJ1*. Therefore, we have sequenced *APJ1* from four bioethanol production strains (Ethanol Red = ER, CAT1, PE2 and VR1) and one commercially available wine strain (ES2). These results showed several additional polymorphisms compared to S288c in the putative promoter and terminator and in the ORF, even causing amino acid changes (Fig. 17). Those changes resulted in seven different *APJ1* alleles. The sequencing results showed that Ethanol Red and ES2 have the same heterozygous alleles of *APJ1*. All industrial strains were heterozygous for *APJ1* except VR1. We constructed single and double *APJ1* deletion strains in the industrial yeast strains ER, ES2, CAT1 and PE2 and scored their growth in 2-fold dilutions on YP plates with different ethanol concentrations. Single deletion of *APJ1* in the different strains did not result in improvement of growth on plates with high ethanol concentrations. We have to note that we did not verify which allele was knocked-out because our main interest was in the ethanol tolerance of the double deletion strains. The double *APJ1* deletion strains showed higher ethanol tolerance compared to the wild type and single deletion strains on YP plates with 16% (v/v) ethanol but the effect was small. The exception was CAT1 where there was no effect at all (Fig. 18). CAT1 has two completely different alleles of *APJ1* compared to the other industrial strains and S288c. Therefore, we cannot rule out that those alleles have already a very low expression level and that deletion of *APJ1* has no further significant effect. In addition, it is also possible that we could not detect any further improvement in ethanol tolerance because CAT1 has already a relatively high ethanol tolerance, just like the VR1-5B strain.



	5'-UTR							APJ1-ORF				3'-UTR						
	-508	-392	-385	-336	-321	-310	-41	123	152	416	524	+87	+106	+116	+121	+270	+409	+449
BY4741	T	A	C-	C	T	C	A	A	S	P	D	T	C	C	G	A	G	A
VR1-5B	C	A	--	G	T	T	G	A	S	P	D	-	C	T	A	A	T	A
ER-1A	C	A	CC	C	T	T	G	T	N	P	N	-	G	T	A	A	T	A
ER-1C	C	A	CC	C	T	T	A	T	N	P	D	-	C	T	A	A	T	A
PE2-1A	C	A	--	G	T	T	G	A	S	P	D	-	C	T	A	A	T	A
PE2-1B	C	A	--	G	T	T	G	A	S	S	D	T	C	C	G	-	T	T
ES2-1A	C	A	CC	C	T	T	G	T	N	P	N	-	G	T	A	A	T	A
ES2-1B	C	A	CC	C	T	T	A	T	N	P	D	-	C	T	A	A	T	A
CAT1-1B	T	A	CC	C	T	T	A	A	S	S	D	T	C	C	G	-	T	T
CAT1-1C	T	C	CC	C	A	T	A	A	S	P	D	T	C	C	G	A	G	A

Figure 17: Sequence analysis of APJ1 *from different industrial yeast strains.* APJ1 *with 550 bp upstream (5'-UTR) and 500bp downstream regions (3'-UTR) of the ORF was sequenced in one complete tetrad from five industrial strains and compared with the BY4741-allele. In total seven different* APJ1 *alleles containing different combinations of polymorphisms were identified.*

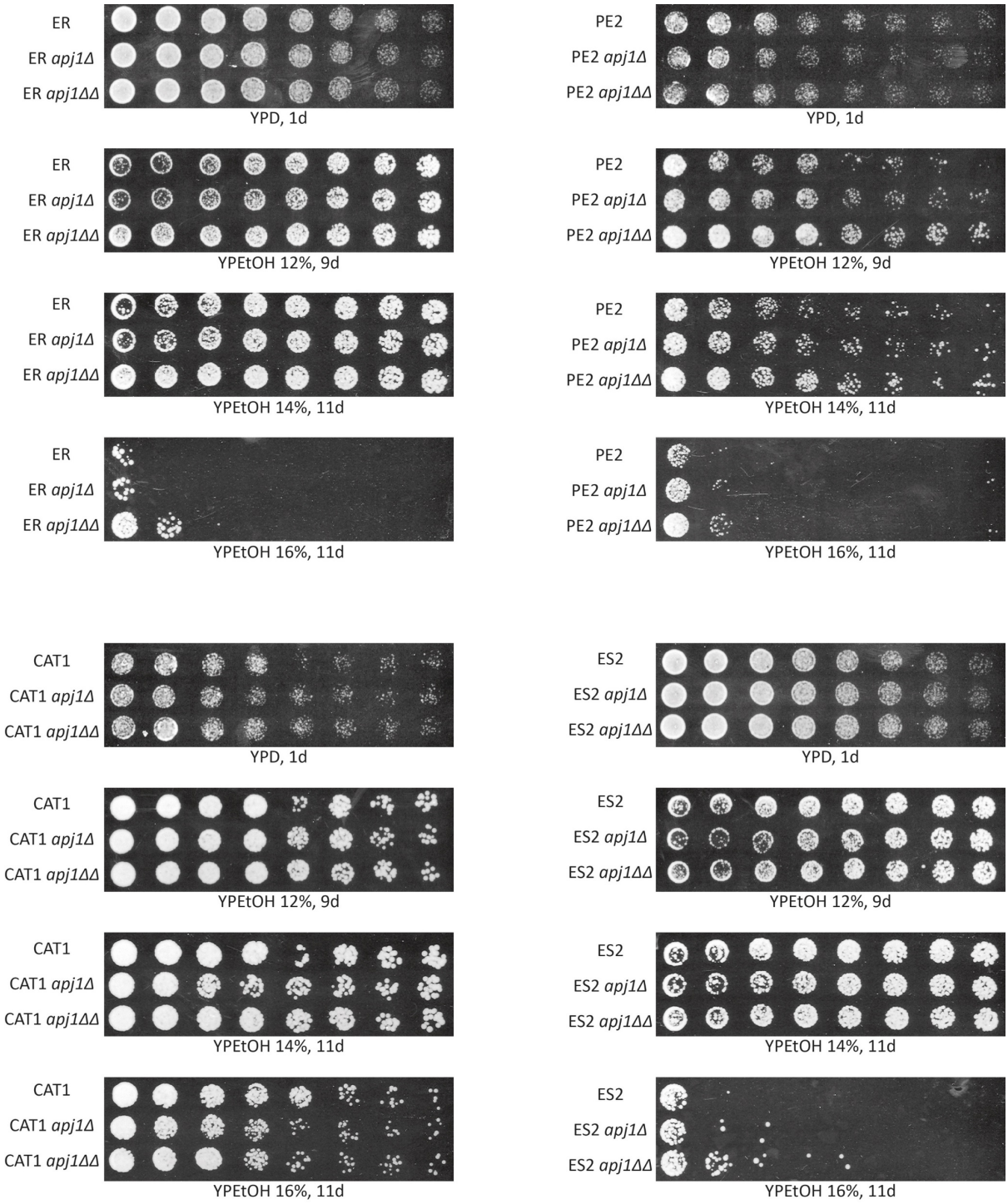


Figure 18: Ethanol tolerance of industrial yeast strains, Ethanol red (ER), PE2, CAT1 and ES2, and its single and double APJ1 deletion strains was determined by scoring growth of 2-fold dilutions on YP plates containing different ethanol concentrations (12%, 14% and 16% (v/v)) at the times indicated.

2.4. Introduction of the different superior alleles in the inferior parent background

After downscaling the three major QTLs and identifying the causative alleles for high ethanol tolerance in the VR1-5B strain, we investigated their contribution and synergy for high ethanol tolerance in the S288c strain with low ethanol tolerance. We have identified as causative genes: *URA3* (QTL1); *VPS70* (QTL2); *MKT1*, *SWS2*, *APJ1* (QTL3). The superior alleles of *URA3*, *VPS70*, *MKT1* and *APJ1* are derived from the VR1-5B background, whereas *SWS2* is a superior allele derived from the inferior parent. Additionally, we found that all auxotrophic mutations were linked to high ethanol tolerance and that even deletion of *APJ1* was beneficial for the phenotype. Therefore, we focussed on the combinations of the causative alleles $MKT1^{VR1\text{-}5B}$, $VPS70^{VR1\text{-}5B}$ and *apj1Δ* in the S288c background.

We have constructed yeast strains with different combinations of the superior alleles in the inferior background using a two-step genetic marker-free approach mediated by the Phi-C31 integrase system. First, the original allele was deleted by introducing an antibiotic resistance marker flanked with attB-attP sites. In a second step the superior allele fused with another antibiotic resistance marker flanked with attB-attP sites was introduced at the original locus in the genome. In the final step, the antibiotic marker was removed using the Phi-C31 integrase leaving a small scar behind the region concerned in the genome. We used the laboratory strain S288c (*Mat**a***) as our starter strain to construct the different combinations of superior alleles. S288c (*Mat**a***) is the prototrophic strain of the inferior parent BY4741 and was used in order to eliminate possible interference with auxotrophic mutations. In this background, the superior allele $SWS2^{BY4741}$ is already present. Growth of the constructed strains was compared with the growth of the BY4741 (inferior) and VR1-5B (superior parent) strains on YPD medium containing different concentrations of ethanol (10% to 16% (v/v)) (Fig. 19). In this spot assay, it was observed that *MKT1* displayed the highest contribution to ethanol tolerance in the S288c background, followed by *VPS70* and *apj1Δ*. The triple combination of the superior alleles *MKT1*, VPS70 and *apj1Δ* showed the best improvement for growth on YPD plates with high ethanol concentrations compared to the single gene replacements and other combinations, even better than the superior parent VR1-5B. It is reasonable to assume that the better growth phenotype of the triple combination strain compared to the superior parent is due to the presence of the superior allele of *SWS2* and/or other mutations which are not present in the VR1-5B strain.

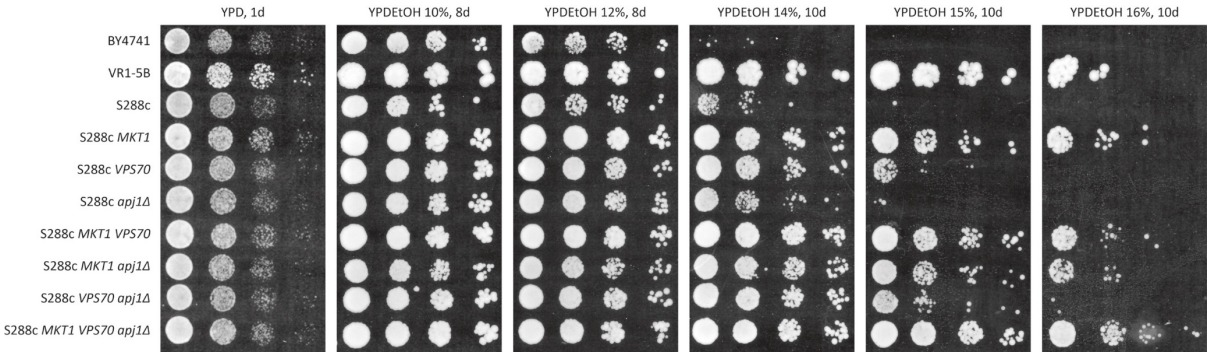


Figure 19: Ethanol tolerance of S288c engineered with different combinations of the superior alleles MKT1, VPS70, and deletion of APJ1 compared to the VR1-5B (superior) and BY4741 (inferior) strains. Ethanol tolerance was tested by spotting 10-fold dilution suspensions on YPD plates containing different concentrations of ethanol (% v/v) and incubating the plates at 30°C for the times indicated.

3. Discussion

Auxotrophic mutations affect tolerance to high concentrations of ethanol (Swinnen *et al.*, 2015). Previous studies have never linked auxotrophic mutations to ethanol tolerance, however those studies were only performed with low to moderate ethanol concentrations (Auesukaree et al., 2009; Fujita et al., 2006; Teixeira et al., 2009; van Voorst et al., 2006). We also found that auxotrophic mutations are not disadvantageous for growth in the presence of low to moderate ethanol concentrations, and that they can even improve growth. Auxotrophic mutations reduce high ethanol tolerance but to a different extent. We have studied both uracil and leucine auxotrophic mutant strains. In our study, we have carried out uptake assays of both uracil and leucine, because the plasma membrane is generally considered as the primary target of ethanol toxicity and uptake of amino acids (Ferreras et al., 1989), acetic acid (Casal et al., 1998), and glucose (Sousa et al., 1995) was reported as being inhibited by ethanol.

Probably, low to moderate ethanol concentrations have little or no effect on nutrient uptake across the plasma membrane and therefore auxotrophic mutations have previously never been linked to ethanol tolerance. Several researchers have called for cautiousness when using auxotrophic strains in genetic experiments (Çakar et al., 1999; Pronk, 2002). Interestingly, our results indicate that the auxotrophic mutations were not disadvantageous for tolerance to

the other stress factors tested, such as acetic acid at pH 5, hydrogen peroxide and high temperature, except for osmotic stress caused by either high glucose or high NaCl concentrations. Hence, osmotic stress might have a similar inhibiting effect on the uptake of auxotrophic nutrients as high ethanol stress. Moreover, when an uracil auxotrophic strain was grown in minimal medium with a limited amount of uracil, not only high ethanol and high salt concentrations but also high temperature was inhibitory. This suggests that stress factors inhibit the growth of auxotrophic strains depending on the growth conditions used by reducing the uptake of the auxotrophic nutrient to a level below what is required for a regular growth rate under the specific growth conditions used.

Swinnen and co-workers (2015) have shown that the *URA3* gene displays much stronger linkage to very high ethanol tolerance than the amino acid biosynthesis genes *HIS3*, *LEU2*, *MET15* and *LYS2*. This suggests that uracil uptake in yeast is more sensitive to high ethanol concentrations than the uptake of amino acids. Our results confirmed that the uptake rate of uracil and leucine is reduced in the presence of increasing ethanol concentrations. Although the uptake rate of uracil was less affected by high ethanol concentrations than that of leucine, the absolute activity of uracil uptake was 500-fold lower than that of leucine. This may explain why *ura3Δ* mutants are more sensitive to ethanol than *leu2Δ* mutants, since uracil uptake activity may more easily drop to levels limiting growth. Fur4 is the only carrier for uracil uptake in yeast, while amino acids are taken up by multiple amino acid permeases. Since natural yeast strains are prototrophic for uracil, uptake of uracil by Fur4 might be an auxiliary, dispensable function, which would explain the very low uptake rate (Jund et al., 1988). Amino acid uptake seems to be much more important for yeast. It is actually essential in media without ammonium and also limits growth in media with low levels of ammonium (Regenberg et al., 1999). In conclusion, our results indicate that very high ethanol concentrations generally comprise the uptake of nutrients required to fulfil auxotrophic requirements, and that therefore auxotrophic mutations lead to lower ethanol tolerance.

In our work, we have also further analysed a major QTL (QTL2) previously identified by Swinnen and co-workers (2012). In this section, QTL2 (chr. X) has been downscaled to gene level by fine-mapping using allele-specific PCR followed by sequence analysis of the centre of the QTL. After RHA analysis of the candidate genes in the centre of this QTL, the *VPS70* allele

of the superior parent VR1-5B was identified as the causative allele for high ethanol tolerance. Both parental strains had active alleles and *VPS70* deletion strains only show reduced tolerance at high ethanol concentrations. This might be the reason why *VPS70* has never been identified in genomic screens for ethanol sensitivity in which generally only low ethanol concentrations have been used. *VPS70* only contributes to the high ethanol tolerance phenotype.

The gene *VPS70* encodes an uncharacterized protein involved in vacuolar protein sorting (Bonangelino et al., 2002). It is localized in the endoplasmic reticulum (Yofe et al., 2016). Another group identified a mutation in an evolved ethanol-tolerant strain, $VPS70^{C595A}$, that provided the largest fitness increase under high ethanol conditions (Voordeckers et al., 2015). The mutation alters the same amino acid as the mutation identified in our superior allele, when compared to the BY background. Moreover, different genomic screens for ethanol tolerance commonly report genes associated with vacuole function. These include genes involved in the vacuolar protein sorting machinery, genes required for assembly of the vacuolar H^+-ATPase complex and genes involved in vesicular transport to the vacuole as being important for ethanol tolerance. Until now the exact molecular mechanism through which the vacuole plays a role in ethanol tolerance is still unclear. However, it is clear that a functional vacuolar system is required for yeast to withstand high ethanol due to the fact that intracellular protein trafficking through endosomes and the vacuole is important for ethanol tolerance. Vacuolar morphology and endocytosis are altered in yeast cells exposed to ethanol stress (Meaden et al., 1999). It is also corroborated that yeast cells exhibit increased vacuolar acidification during ethanol stress and this has been proposed as one of the recovery mechanisms from stress-induced cytosolic acidification (Martinez-Munoz and Kane, 2008).

In the present study, we have also investigated the relevance of the *APJ1* gene, which was identified by RHA in the BY4741/VR1-5B background as one of the three causative genes in QTL3 on chromosome XIV and contributed to the high ethanol tolerance phenotype of VR1 (Swinnen et al., 2012). *APJ1* encodes a putative chaperone of the Hsp40/DnaJ family. These proteins are involved in regulation of Hsp70 chaperone activity by stimulating the intrinsically weak ATPase activity of the Hsp70 proteins and facilitating Hsp70 interaction with denaturated proteins (Cyr et al., 1994). Apj1 functions in the proteolysis of proteins targeted

for degradation by sumoylation as part of a larger Hsp70-based chaperone machinery (Sahi et al., 2013). By comparing the *APJ1* sequence in the VR1-5B and BY4741 strains, Swinnen and co-workers (2012) noticed that the *APJ1* alleles encoded identical proteins but contained several mutations in the promoter and terminator regions. Moreover, we observed that deletion of *APJ1* enhanced ethanol tolerance, although deletion of *APJ1* in the VR1-5B background caused less improvement in ethanol tolerance than in the BY4741 background. *APJ1* expression, especially in the beginning of the fermentation, was about 2.5-fold higher in the BY4741 strain than in the VR1-5B strain. Although the conditions of the expression analysis and growth experiments are different, this suggests that Apj1 has a negative effect on ethanol tolerance and that the link to the superior parent VR1-5B is due to its lower expression in that strain. We also tried to improve ethanol tolerance in several industrial strains that have different *APJ1* alleles. Our results show that not all strains could be improved in their ethanol tolerance by only deleting the *APJ1* gene, which means that changing one genetic determinant does not necessarily lead to the improvement of a phenotype, especially when it is a polygenic trait.

After identification of all the causative genes from the major QTLs that contribute to the high ethanol tolerance phenotype of the VR1 strain, the contribution and synergy of the different superior alleles was determined in the inferior BY4741 strain. From these results, we can state that *MKT1* makes the highest contribution to ethanol tolerance in the S288c background followed by *VPS70* and *apj1Δ*. Surprisingly, the triple combination of the superior alleles *MKT1*, *VPS70* and *apj1Δ* in the S288c strain even supported a higher ethanol tolerance than that of the superior parent VR1-5B, which indicates that the presence of the superior allele *SWS2* from the inferior parent and/or other mutations in this background could be beneficial for ethanol tolerance.

In conclusion, our results indicate that very high ethanol concentrations generally comprise the uptake of nutrients required to fulfil auxotrophic requirements and that therefore auxotrophic mutations may lead to lower ethanol tolerance. We have identified *VPS70* as a new causative allele in QTL2 that contributes to the high ethanol tolerance phenotype of the industrial yeast strain VR1. Additionally, we confirmed that *APJ1* has a negative effect on ethanol tolerance and that a lower expression or deletion of the *APJ1* allele improves ethanol

tolerance. Finally, we introduced all superior alleles in the inferior background and succeeded in improving ethanol tolerance of the inferior parent strain to an even higher level of that in the superior parent.

4. Own publications on these subjects

- Duitama*, J., A. Sanchez-Rodriguez*, A. Goovaerts*, S. Pulido-Tamayo, G. Hubmann, M. R. Foulquie-Moreno, J. M. Thevelein, K. J. Verstrepen and K. Marchal (2014). Improved linkage analysis of Quantitative Trait Loci using bulk segregants unveils a novel determinant of high ethanol tolerance in yeast. BMC Genomics *15*: 207.

  **Shared first author*

- Swinnen, S., Schaerlaekens, K., Pais, T., Claesen, J., Hubmann, G., Yang, Y., Demeke, M., Foulquie-Moreno, M.R., Goovaerts, A., Souvereyns, K.*, et al.* (2012). Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. Genome Res *22*, 975-984.

- Swinnen, S.*, Goovaerts, A.*, Schaerlaekens, K., Dumortier, F., Verdyck, P., Souvereyns, K., Van Zeebroeck, G., Foulquie-Moreno, M.R., and Thevelein, J.M. (2015). Auxotrophic mutations reduce tolerance of *Saccharomyces cerevisiae* to very high levels of ethanol Stress. Eukaryot Cell *14*, 884-897.

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The author participated in the project conception, experimental work, data analysis and article writing.

CHAPTER III

Identification of causative alleles determining maximal ethanol accumulation capacity

1 Introduction

Worldwide, bioethanol has become a renewable and environmentally friendly alternative for fossil fuels. However, the production process of bioethanol is not always cost-competitive with gasoline production (Puligundla et al., 2011). The major costs in the bioethanol production process are those of the biomass substrate and of energy consumption. Hence, applying very high gravity (VHG) fermentations can offer great savings in process water and energy consumption. Fermentation of high initial sugar concentrations (>270 g/L) results in higher final ethanol concentrations in the medium (Bai et al., 2004; Kwiatkowski et al., 2006). However, during VHG fermentation, yeast cells are subjected to a number of stresses such as osmotic stress, heat stress, oxidative stress and particularly high ethanol stress, which negatively affect viability and fermentation productivity leading to sluggish or stuck fermentations. Hence, yeast strains with high ethanol tolerance are important to obtain high ethanol yield and maximal ethanol accumulation capacity (Liu et al., 2012).

In the last decades ethanol tolerance of *S. cerevisiae* has attracted much attention in research, which has broadened our knowledge on the underlying molecular mechanisms. Unfortunately, most previous studies on ethanol tolerance were based on growth and/or survival assays of laboratory yeast strains on nutrient plates or in liquid media in the presence of relatively low ethanol concentrations, which is different from the stress of very high ethanol concentrations encountered in industrial fermentations. During VHG fermentations, the relevance of the ethanol tolerance of the *S. cerevisiae* strain used translates into the maximal ethanol level that the strain can accumulate.

Pais and co-workers (2013) have applied pooled-segregant whole genome sequence analysis to compare the genetic basis of maximal ethanol accumulation capacity and high ethanol tolerance in the sake yeast strain, CBS1585. They showed that both traits have a partially different genetic basis and found several QTLs that were linked to maximal ethanol accumulation capacity. This study identified three genes required for maximal ethanol accumulation capacity, being the superior alleles of the superior parent CBS1585, located in two QTLs: *ADE1* (QTL2, chr. I), *KIN3* (QTL2, chr. I) and *URA3* (QTL3, chr. V). However, these

alleles were not linked in this genetic background to high ethanol tolerance of yeast growth on solid nutrient plates. *KIN3* (QTL2) encodes a protein kinase involved in DNA damage repair (Moura et al., 2010). It is widely recognised that ethanol is a mutagen at least of the mitochondrial DNA, which strengthens the significance of this enzyme for DNA damage repair during alcoholic fermentation (Ibeas and Jimenez, 1997). Ade1 is involved in de novo purine biosynthesis and, even if it has never been connected to ethanol tolerance, several *ADE* genes have been associated with high sugar tolerance in yeast (Ando et al., 2006). *URA3* has also been identified as a causative gene for maximal ethanol accumulation capacity (Pais et al., 2013) and even for high ethanol tolerance of growth on solid nutrient plates (Swinnen et al. 2015). However, in the specific genetic background of the strain CBS1585, *URA3* was not linked to high ethanol tolerance of growth on solid nutrient plates.

In the present study, we report the genetic dissection of two other QTLs linked to maximal ethanol accumulation capacity in order to identify novel ethanol stress-related genes. Our work has identified two new causative alleles sustaining maximal ethanol accumulation, which broadens our understanding of the genetic basis underlying maximal ethanol accumulation capacity of the strain CBS1585.

In addition, Pais and co-workers (Martins Pais, 2012) applied pooled-segregant gene expression analysis in order to identify genes involved in maximal ethanol accumulation capacity and ethanol tolerance. They evaluated genome-wide gene expression at a specific fermentation time point, when the ethanol concentration was 13.8% (v/v) and the glucose leftover 11.5% (w/v), for the superior pool, superior parent Seg5 and the inferior parent BY710. The RNA sequencing data revealed different genes that were overexpressed in the superior pool and superior parent compared to the inferior parent BY710. The biological function of most of these genes has not previously been related to stress tolerance. In the last part of this chapter, we have examined the target genes identified in pooled-segregant gene expression analysis in order to identify genes of which the modification could improve maximal ethanol accumulation capacity in industrial yeast strains.

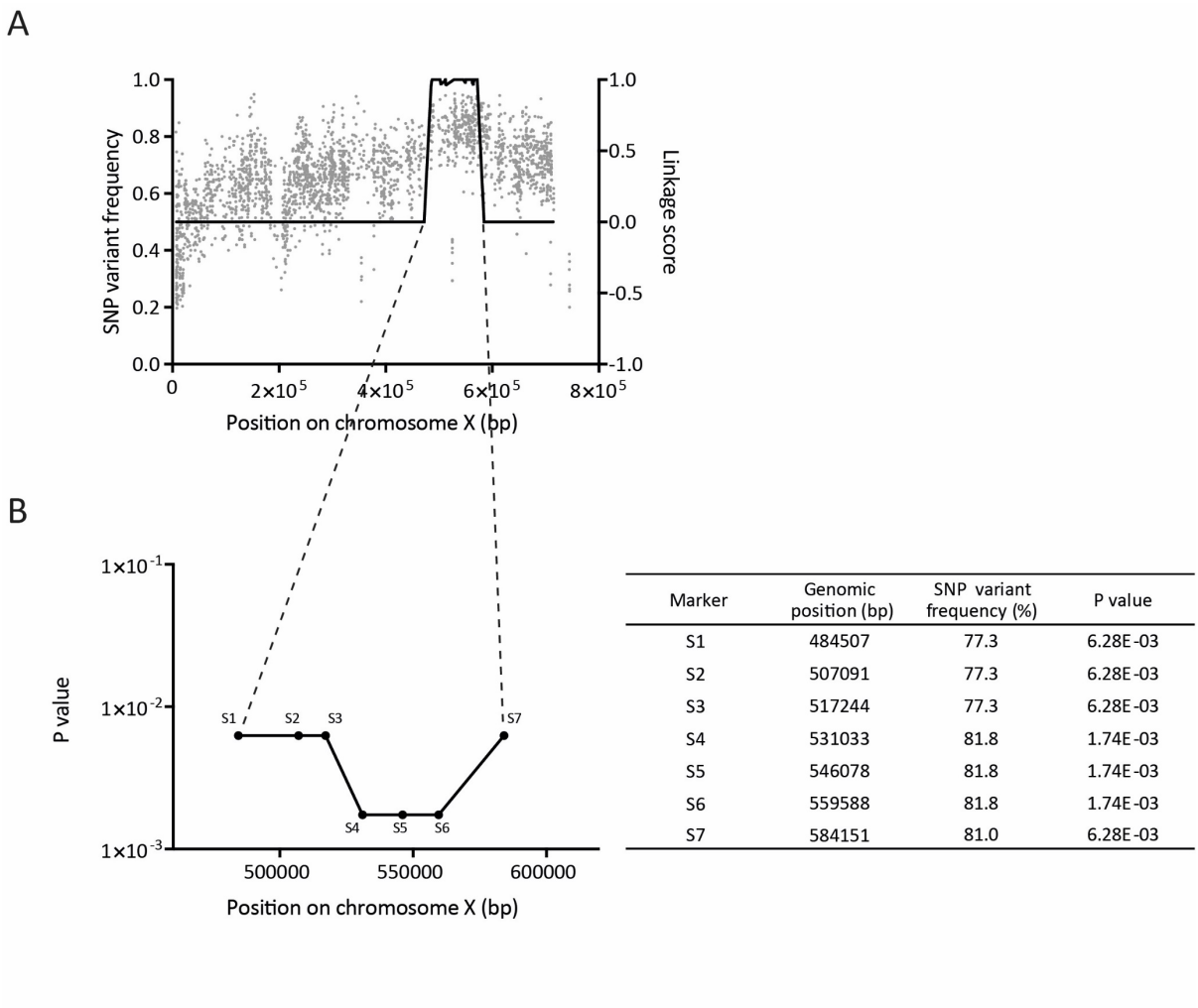
2 Results

2.1 Genetic dissection of QTL9 on chromosome X linked to maximal ethanol production capacity

Pais and co-workers (2013) have performed pooled-segregant whole-genome sequence analysis of maximal ethanol accumulation capacity in fermenting cells in the absence of cell proliferation by pooling the 22 best-performing segregants as determined in semi-static VHG fermentations. Eight QTLs were linked to the superior parent Seg5, a haploid segregant of the sake strain CBS1585, and three QTLs linked to the inferior parent lab strain BY710. BY710 is an artificially marked strain derived from the lab strain BY4742 and has the same genotype *Matα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*. To date, only two QTLs (QTL2 and QTL3) have been further analysed and causative alleles of the genes *ADE1*, *KIN3* and *URA3* have been identified. In this section, we have analysed QTL9 located on chromosome X because this QTL was the next QTL with the strongest linkage to the superior parent Seg5 for maximal ethanol accumulation capacity.

2.1.1 Fine-mapping QTL9 by bulk reciprocal hemizygosity analysis (bulk RHA)

After sequence analysis of the pool, the SNP variant frequency was plotted against the chromosomal position. Visual analysis of the SNP variant frequency significantly deviating from the mean of 0.5 was indicative of genetic linkage to maximal ethanol accumulation capacity. The statistical significance of QTL9 was confirmed using a Hidden Markov Model (HMM) (Duitama et al., 2014). For each SNP, the HMM scores a probability of linkage between -1 and 1; linkage score of 1 indicates link to the superior parent (Seg5) while linkage score -1 indicates link to the inferior parent (BY710). QTL9 located on chromosome X clearly showed a strong linkage to the superior parent Seg5 for maximal ethanol accumulation capacity (Fig. 20A). QTL9 is a large QTL of approximately 110 kb, consisting of 40 genes. Therefore, different SNPs in the 22 individual segregants were scored by allele-specific PCR to determine precisely the SNP variant frequency and the statistical significance of the SNPs within the QTL. P-values were calculated using an exact binominal test with a confidence level of 95% and were plotted over the length of chromosome X. SNP variant frequency, linkage score obtained by HMM analysis and P-values of QTL9 on chromosome X are shown in figure 20.



Marker	Genomic position (bp)	SNP variant frequency (%)	P value
S1	484507	77.3	6.28E-03
S2	507091	77.3	6.28E-03
S3	517244	77.3	6.28E-03
S4	531033	81.8	1.74E-03
S5	546078	81.8	1.74E-03
S6	559588	81.8	1.74E-03
S7	584151	81.0	6.28E-03

Figure 20: QTL9, located on chromosome X, identified with pooled-segregant whole genome sequence analysis as being linked to maximal ethanol accumulation capacity. (A) SNP variant frequency was plotted against the respective chromosomal position. Probability of linkage to the superior parent was determined with HMM and indicated on the right axis. (B) Detailed information about the chromosomal position of the SNPs and their respective P-values determined with allele-specific PCR in the 22 individual segregants of pool 1.

Fine-mapping of the locus based on P-value calculations was not possible because we used a selected pool of only 22 segregants. The difference in P-value of the selected SNPs in figure 20B was due to a difference in only one segregant, making it dangerous to exclude on this basis the other parts of the linked QTL. Another way of fine-mapping a broad QTL is to divide the QTL into blocks in order to evaluate a series of adjacent genes by 'bulk RHA', or bRHA, a modification of the functional assay named reciprocal hemizygosity analysis (RHA), which evaluates multiple genes together rather than single genes one by one (Steinmetz et al., 2002). We have divided QTL9 into 4 blocks of approximately 25 kb (Appendix 1). The heterozygous strains were constructed by deletion of a whole block of adjacent genes using the antibiotic marker KanMX in the hybrid Seg5/BY4742. Allele-specific PCR was performed

in order to identify which allele of the block had been kept and which one deleted in the hybrid, either the block derived from the superior parent Seg5 or that from the laboratory strain BY4742. The two reciprocally deleted heterozygous strains for each block were evaluated in VHG fermentations using YP + 33% (w/v) glucose to address the effect of the Seg5 and BY4742 alleles on maximal ethanol accumulation capacity. The results showed no difference in maximal ethanol accumulation for block 1, 2 and 4 suggesting none of these blocks contains a causative gene. In the third block of genes, however, there was a clear reduction in maximal ethanol accumulation when the allele block from the Seg5 strain was deleted compared to deletion of the allele block from BY4742 (Fig. 21A). In this way, we could narrow down the QTL size from 110 kb to 26 kb. Block 3 contained 13 genes that nearly all had polymorphisms either in the ORF, promoter or terminator region. Hence, we decided to divide this block in 4 sub-blocks of approximately 8 kB (Appendix 1) and applied once more bRHA methodology to evaluate the new reciprocal strains in VHG fermentation for maximal ethanol accumulation capacity. The bRHA analysis showed a significant difference between the reciprocal strains of sub-block 3.3 for maximal ethanol accumulation capacity (Fig. 21B). This indicated the presence of one or more causative alleles in this region. Sub-block 3.3 was approximately 9 kb in length and consisted of four genes, two tRNAs and one ARS sequence (Fig. 21C). The four genes were *YJR056C*, *CDC8*, *APS2* and *PTK2*. *YJR056C* encodes a protein of unknown function; *CDC8* encodes a thymidylate and uridylate kinase which converts dTMP into dTDP and dUMP into dUDP (Jong and Campbell, 1984); *APS2* encodes a small subunit of the clathrin-associated adaptor complex AP-2, which is involved in protein sorting at the plasma membrane (Kirchhausen et al., 1991) and *PTK2* encodes a serine/threonine protein kinase involved in the regulation of Pma1 (plasma membrane H^+-ATPase) (Eraso et al., 2006). In order to identify the causative allele in this sub-block 3.3, we investigated the most likely candidate gene individually with RHA. The two tRNAs and ARS sequence were excluded from evaluation because their involvement appeared to be unlikely. All four genes had polymorphisms within their ORF and/or promoter and terminator region (Fig 21C). However, only *PTK2* showed nonsynonymous substitutions (Table 4). Therefore, it was evaluated by RHA, which showed a clear reduction of maximal ethanol accumulation capacity in VHG fermentation when the Seg5 allele was deleted compared to deletion of the BY allele, indicating that *PTK2* was a causative gene for maximal ethanol accumulation capacity in Seg5 (Fig 21D).

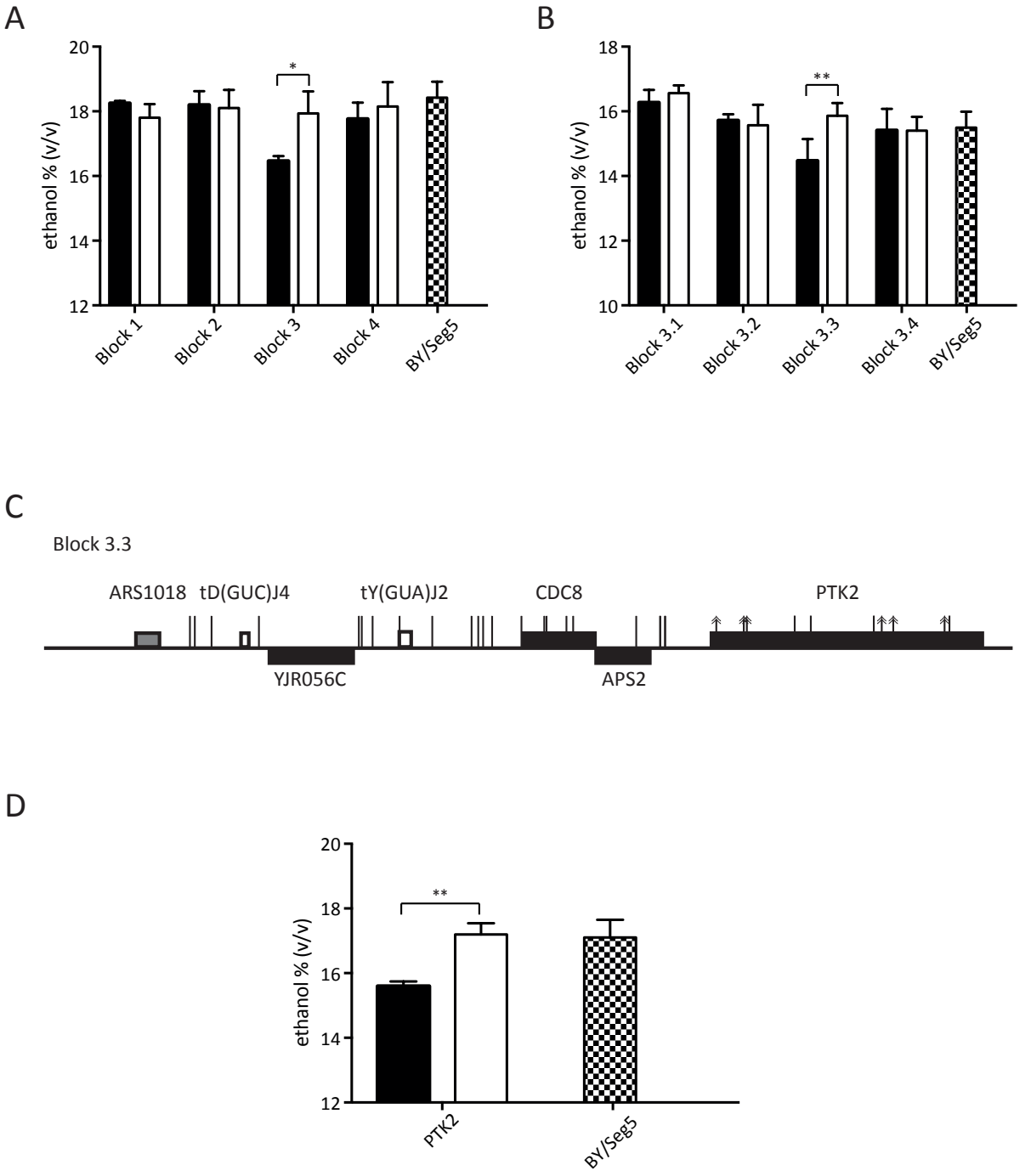


Figure 21: Analysis of QTL9 (chr. X) resulting in identification of the causative gene PTK2. (A) Final ethanol concentration in VHG fermentation with the bRHA strains of QTL9. (B) Final ethanol concentration in VHG fermentation with the bRHA strains of the sub-blocks in block 3 of QTL9. (C) Genes present in block 3.3 are shown as annotated in the Saccharomyces Genome Database. Mutations (I) and nonsynonymous mutations (↟) are also indicated. (D) Final ethanol concentration in VHG fermentation with the RHA strains for the PTK2 gene. Black bars represent hybrid strains with the Seg5 allele or block of alleles deleted, white bars represent hybrid strains with the BY allele or block of alleles deleted, shaded bars represent the control hybrid strain BY/Seg5. The results were analysed with student T-test. (Statistical significance identifies p-values between 0.01 and 0.05 (*), between 0.01 and 0.001 (**).

Table 4: Occurrence of SNPs and amino acid changes in the causative gene PTK2 *and its gene product, respectively, between S288c and Seg5*

	Promoter	ORF *PTK2*									
Position	**-181**	**50**	**292**	**317**	**741**	**873**	**1414**	**1489**	**1592**	**2027**	**2034**
S288c	G	G	T	T	T	C	C	G	A	A	C
Seg5	A	A	C	C	C	T	T	C	T	T	A
Amino acid change in Ptk2		N(17)S	P(98)S	A(106)V				L(497)V	M(531)K	M(676)K	

2.2 Genetic dissection of QTL17 on chromosome XV linked to maximal ethanol accumulation capacity and high ethanol tolerance of cell growth

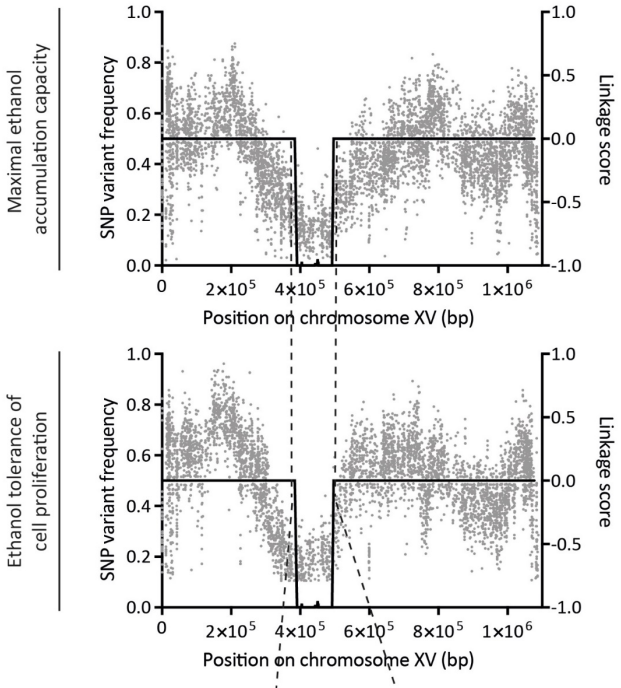
Pais and co-workers (2013) have also applied pooled-segregant whole genome sequencing analysis in order to investigate tolerance of cell proliferation to high levels of ethanol by pooling 32 superior segregants originating from a cross between Seg5 and an artificially marked BY4742 strain (BY710). Segregants were screened for growth on YPD medium containing 18 to 20% (v/v) ethanol. Twelve segregants were common between the superior pool with high ethanol tolerance of cell proliferation and the pool of maximal ethanol accumulation capacity. QTL analysis of both polygenic traits identified two common QTLs, concerning the common character of those QTLs, because the two QTLs might contain a different causative gene for each trait. In the next section, we discuss the genetic dissection of QTL17 on chromosome XV, a strong QTL that was common to both polygenic traits under investigation and, in contrast to other QTLs, linked to the inferior parent BY710.

2.2.1 Fine-mapping QTL17 by bulk reciprocal hemizygosity analysis (bulk RHA)

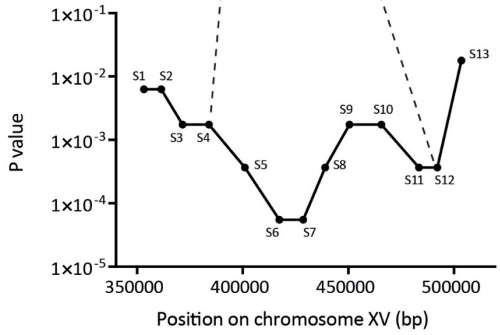
The statistical significance of QTL17 was confirmed using a hidden Markov model (HMM) (Duitama et al., 2014) for both polygenic traits (Fig. 22). QTL17 is a large QTL of approximately 130 kb, which consists of many genes. We have scored multiple SNPs in the 22 individual segregants pooled for maximal ethanol accumulation capacity by allele-specific PCR to determine precisely the SNP variant frequency and the statistical significance of the QTL. P-values were calculated using an exact binominal test with a confidence level of 95% and were plotted over the length of chromosome XV. SNP variant frequency, linkage score obtained by

HMM analysis for both pools and P-value of the SNPs scored in QTL17 on chromosome XV are shown in figure 22.

A



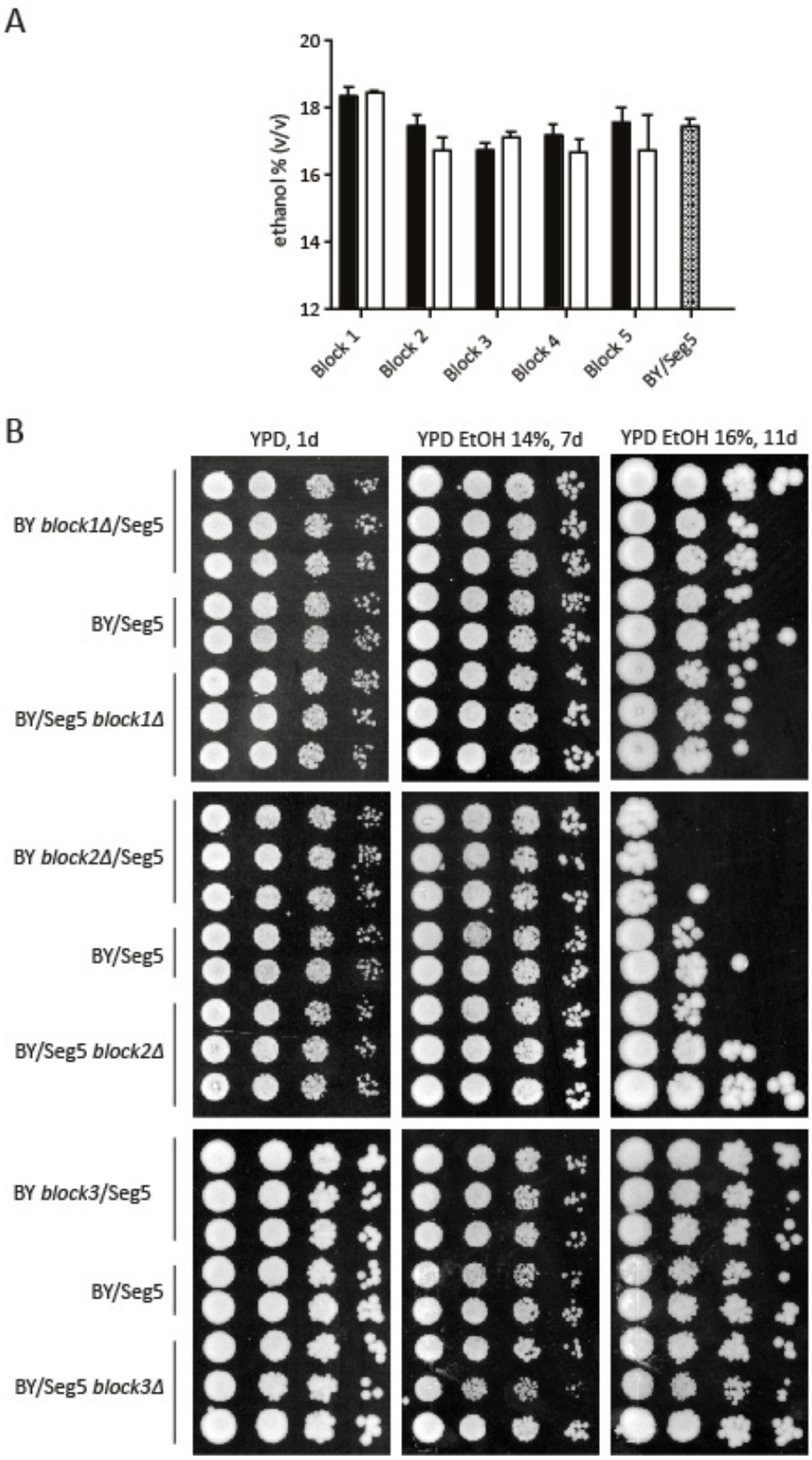
B



Marker	Genomic position (bp)	SNP variant frequency (%)	P value
S1	353226	22.7	6.28E-03
S2	361461	22.7	6.28E-03
S3	371543	18.2	1.74E-03
S4	384077	18.2	1.74E-03
S5	401076	13.6	3.67E-04
S6	417371	9.1	5.51E-05
S7	428615	9.1	5.51E-05
S8	439005	13.6	3.67E-04
S9	450502	18.2	1.74E-03
S10	465693	18.2	1.74E-03
S11	483466	13.6	3.67E-04
S12	492191	13.6	3.67E-04
S13	503561	27.3	1.78E-02

Figure 22: QTL17, located on chromosome XV, identified with pooled-segregant whole genome sequence analysis for maximal ethanol accumulation capacity and ethanol tolerance of cell proliferation. (A) SNP variant frequency was plotted against the respective chromosomal position. Probability of linkage to the superior parent was determined with HMM and indicated on the right axis. (B) Detailed information about the chromosomal position of the SNPs and their respective P-values determined with allele-specific PCR in the 22 individual segregants of pool 1.

Fine-mapping of the locus based on P-value calculations showed that the strongest linkage of the QTL was between SNP markers S6 and S7. However, as stated in the previous section, we could not exclude that a causative gene was outside of that range because of the small number of segregants. Therefore, we proceeded with fine-mapping the QTL by dividing the QTL into different blocks in order to apply 'bulk RHA'. We divided QTL17 into 5 blocks of approximately 25 kb (Appendix 2). Two reciprocally deleted hemizygous diploid strains were constructed for each block and the strains were evaluated in VHG fermentations with YP + 33% (w/v) glucose to evaluate the effect of the Seg5 and BY4742 alleles on maximal ethanol accumulation capacity and ethanol tolerance of cell proliferation on YPD medium with 14% and 16% ethanol (v/v). The results of bRHA for maximal ethanol accumulation suggested only for block 2 a very small contribution, that was however not statistically significant. This indicated that apparently none of the blocks contained a causative gene for this trait (Fig. 23A). However, the results of the growth assay on YPD plates in the presence of high ethanol concentrations showed a clear difference in ethanol tolerance between the two diploid strains carrying block 2. The strain with block 2 derived from BY4742 grew very well in the presence of 16% (v/v) ethanol, whereas the reciprocal strain with the block from Seg5 did not grow at all. Moreover, the heterozygous strain containing block 2 of BY4742 grew even better than the control hybrid, which suggested that block 2 contained a gene of which the allele from the superior parent had a negative effect on ethanol tolerance (Fig. 23B). In that way, we could narrow down the QTL size from 130 kb to 28 kb at least for the trait of high ethanol tolerance of cell proliferation. Block 2 contained 18 genes that nearly all had polymorphisms either in the ORF, promoter or terminator region.


A
ethanol % (v/v)
20
18
16
14
12
Block 1
Block 2
Block 3
Block 4
Block 5
BY/Seg5
B
YPD, 1d
YPD EtOH 14%, 7d
YPD EtOH 16%, 11d
BY block1Δ/Seg5
BY/Seg5
BY/Seg5 block1Δ
BY block2Δ/Seg5
BY/Seg5
BY/Seg5 block2Δ
BY block3/Seg5
BY/Seg5
BY/Seg5 block3Δ

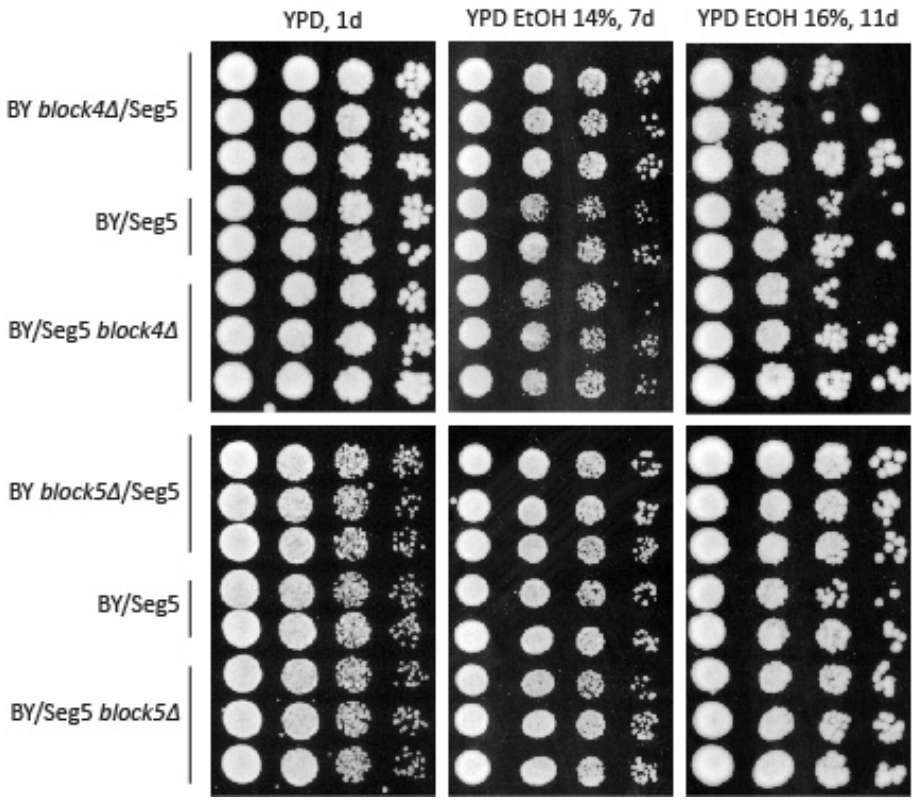


Figure 23: Analysis of QTL17 on chromosome XV by bRHA. (A) Final ethanol concentrations during VHG fermentation of the bRHA strains of QTL17. Black bars represent hybrid strains with Seg5 allele block deleted, white bars represent hybrid strains with BY allele block deleted, shaded bar represents the control strain BY/Seg5. (B) Determination of ethanol tolerance of cell proliferation of the bRHA strains of QTL17 by scoring growth of tenfold dilutions on 14 and 16% (v/v) ethanol after the indicated number of days.

Instead of further subdividing block 2 in sub-blocks, as a proof of principle, we have applied a new methodology through Gnomixx, a consultancy company founded by Dr Vuylsteke, to re-evaluate the statistical analysis of this QTL17. It was found that the strongest linkage on chromosome XV was around the chromosomal position 400700, which lies in block 2 of QTL17. Hence, we decided to investigate a region of 5 kb around this indicative position. This region contained three genes *SHE4*, *PEP12* and *CYC2*. *SHE4* encodes a protein that regulates myosin function and hereby polarization of the actin cytoskeleton (Toi et al., 2003); *PEP12* encodes a target membrane receptor (t-SNARE) that controls vesicles travelling between Golgi apparatus and the vacuole (Gerrard et al., 2000); and *CYC2* encodes a membrane bound flavoprotein that participates in ligation of heme to mitochondrial c-type cytochromes (Bernard et al., 2005). We investigated those three genes individually by RHA and evaluated the reciprocal strains in VHG fermentation for maximal ethanol accumulation capacity and in growth assay for ethanol tolerance (Fig. 24). The heterozygous strains obtained by deleting the specific alleles of the *SHE4* gene revealed a clear difference in the maximal ethanol concentration at the end of the fermentation. (Fig. 24A). Moreover, the phenotypic analysis of growth on YPD plates with high ethanol concentrations showed also a clear difference in ethanol tolerance between the same two heterozygous strains, carrying a different allele of *SHE4* (Fig. 24B). The presence of the BY4742 allele of the *SHE4* gene resulted in a positive contribution to both traits. Reciprocal strains for *PEP12* and *CYC2* did not show any difference for ethanol accumulation during fermentation or ethanol tolerance (Fig. 24). The results indicate that *SHE4* from BY4742 is a causative allele in QTL17 for maximal ethanol accumulation in fermentations and ethanol tolerance on plates. Sequence analysis of the *SHE4* allele revealed three SNPs in the promoter, twenty SNPs in the ORF, changing four amino acids in the sequence of the gene product, and four SNPs in the terminator region (Table 5). These amino acid changes have been checked in 44 yeast strains of which the whole genome sequence has been published. 7 yeast strains (UWOPSO5_217_3, T7, YPS128, YPS163, UC5, K11, Kyokai7 and Jay291) had the same inferior *SHE4*-allele as the Seg5 strain. Those strains were either natural isolates, sake or bioethanol strains. Therefore, the *SHE4*-allele of BY4742 seems to be a good candidate for industrial strain improvement.

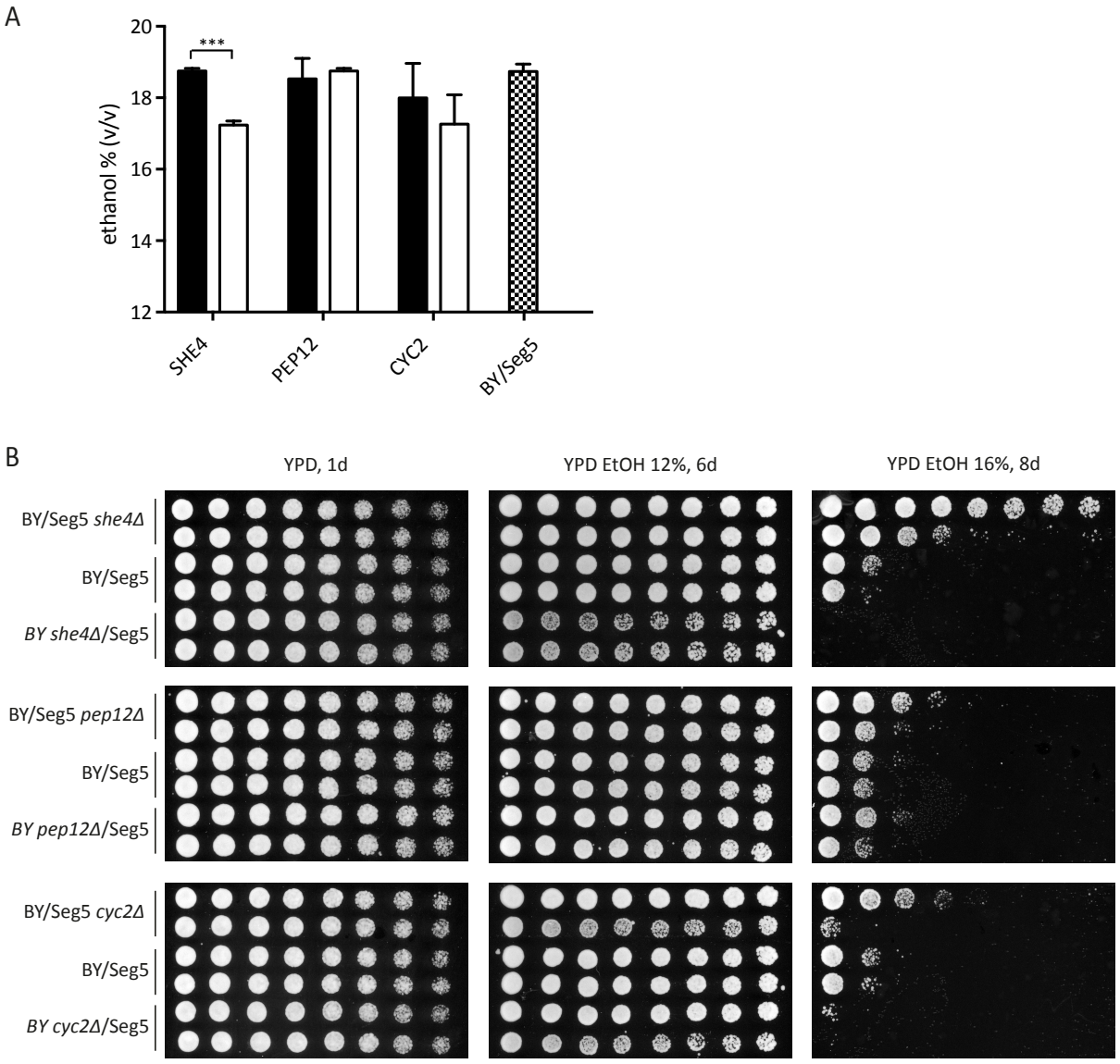


Figure 24: Analysis of three genes located in the region with the strongest linkage of QTL17 by RHA. (A) Final ethanol concentration in VHG fermentations of the RHA strains. Black bars represent the hybrid strain with the Seg5 allele deleted, white bars represent the hybrid strain with the BY allele deleted, the shaded bar represent the control strain BY/Seg5. The results were analysed with student T-test. (Statistical significance identifies p-values between 0.01 and 0.05 (*), between 0.01 and 0.001 (**), and 0.001 and 0.0001 (***)). (B) Determination of ethanol tolerance of cell proliferation in RHA strains by scoring the growth of twofold dilutions in the presence of 12 and 16% (v/v) ethanol after the indicated number of days.

Table 5: Occurrence of SNPs and amino acid changes in the causative gene SHE4 *and its gene product, respectively.*

	Promoter			ORF *SHE4*						
position	**-213**	**-149**	**-3**	**6**	**33**	**66**	**83**	**84**	**111**	**451**
S288c	A	A	A	A	C	A	A	A	T	A
Seg5	G	T	G	G	T	G	C	T	G	G
Amino acid change in She4							K(28)T		N(37)K	I(151)V

	ORF *SHE4*									
position	**579**	**747**	**1020**	**1089**	**1104**	**1188**	**1440**	**1500**	**1512**	**1638**
S288c	A	A	A	A	G	T	G	C	A	T
Seg5	G	T	G	G	A	A	A	T	G	C
Amino acid change in She4		K(249)N								

	ORF *SHE4*			Terminator			
position	**1689**	**1863**	**2082**	**2498**	**2612**	**2613**	**2615**
S288c	A	G	C	C	C	C	T
Seg5	G	A	T	T	-	-	C
Amino acid change in She4							

2.3 Applicability of overexpression of target genes to improve maximal ethanol accumulation capacity in an industrial yeast strain

Pais and co-workers have performed comparative transcriptomic analysis using micro-array genome-wide gene expression analysis and RNA sequencing to determine the transcriptional response in *S. cerevisiae* to ethanol at a certain time-point during fermentation and to identify target genes for improvement of maximal ethanol accumulation capacity. Samples of the pool of segregants, the superior parent Seg5, and inferior parent BY710 were taken at a certain time-point in VHG fermentation (YP + 35%, w/v glucose; 200 rpm, 25°C), in which glucose leftover was around 11.5% (w/v), ethanol concentration was around 13.8% (v/v) and cell viability was still more than 60%. For more details about the experimental set-up, we refer to the doctoral dissertation of Thiago M. Pais (Martins Pais, 2012). Combination of the micro-array gene expression analysis and RNA sequence analysis revealed a top list of 37 genes overexpressed (log >1.5) in the pool of segregants and the superior parent Seg5 compared to the inferior parent BY710 (Table 6). They constitute candidate genes for improving ethanol accumulation in very high gravity fermentation.

Table 6: List of genes most highly overexpressed in the pool of segregants and the superior parent Seg5 compared to the inferior parent BY710.

Gene/ORF	Description
YGL101W	Biological function unknown
YDL218W	Biological function unknown
YMR175W-A	Biological function unknown
CYB5	Act as an electron donor involved in the sterol and lipid biosynthesis pathways
CYC1	Electron carrier in the mitochondrial intermembrane space involved in electron transport during respiration
YIL169C	Biological function unknown, similar to HPF1
ROX1	Transcription factor that represses expression of hypoxia-induced genes in the presence of oxygen and represses target genes during osmotic stress
YEH1	Steryl ester hydrolase involved in sterol homeostasis
HPF1	Encodes a haze-protective mannoprotein
POX1	Fatty-acyl coenzyme A oxidase; involved in the fatty acid beta-oxidation pathway
THI73	Putative plasma membrane permease; proposed to be involved in carboxylic acid uptake
RMR1	Protein required for meiotic recombination and gene conversion
CDC123	Assembly factor for the eIF2 translation initiation factor complex; regulates translational initiation
ECM13	Biological function unknown
FMO1	Flavin-containing monooxygenase; involved in maintenance of the ER redox buffer ratio for correct folding of disulphide-bonded proteins
RPI1	Transcription factor which mediates fermentation stress tolerance by modulating cell wall integrity
EDC2	RNA-binding protein that directly activates mRNA decapping; has a role in translation during heat stress
PDR11	ATP-binding cassette (ABC) transporter; mediates sterol uptake when sterol biosynthesis is compromised
YKR045C	Biological function unknown
KNH1	Protein with similarity to Kre9p; Kre9p is involved in cell wall beta 1,6-glucan synthesis
YDL114W	Putative short-chain dehydrogenase/reductase
YOL159C	Biological function unknown
STB5	Transcription factor; involved in oxidative stress response
COX5A	Subunit Va of cytochrome c oxidase; cytochrome c oxidase is the terminal member of the mitochondrial inner membrane electron transport chain
HMG1	HMG-CoA reductase; catalyses conversion of HMG-CoA to mevalonate, involved in sterol biosynthesis
SPE2	Adenosylmethionine decarboxylase involved in pantothenate, spermine and spermidine biosynthesis
LCB5	Sphingosine kinase
SNQ2	Plasma membrane ATP-binding cassette (ABC) transporter
COX8	Subunit VIII of cytochrome c oxidase (Complex IV); involved in the electron transport chain
TRM2	tRNA methyltransferase
YHR175W-A	Biological function unknown
YBR056W-A	Biological function unknown
SIP18	Phospholipid-binding hydrophilin; expression is induced by osmotic stress
ADE1	involved in the biosynthesis of purine nucleotides
KIN3	serine/threonine protein kinase; possible involved in DNA damage response
TIR1	Cell wall mannoprotein
ADH2	NAD-dependent alcohol dehydrogenase that converts ethanol to acetylaldehyde

In order to evaluate the contribution of 35 of those genes to maximal ethanol accumulation capacity, we have cloned those genes from the superior parent Seg5 in the high copy expression vector pVV214-HPH. Two genes, *YEH1* and *HPF1,* were not evaluated due to difficulties with the construction of the expression vectors. The different overexpression plasmids were transformed into the *S. cerevisiae* strain V1116, a robust wine strain, to evaluate their contribution to maximal ethanol accumulation in very high gravity fermentation. The V1116 strain was chosen because it was previously used as a reference strain to evaluate maximal ethanol accumulation capacity under VHG conditions (doctoral thesis of Thiago M. Pais, 2012), and could be improved further for its maximal ethanol accumulation capacity. Moreover, the sake strain CBS1585, diploid parent of the superior haploid strain Seg5, was able to accumulate more ethanol than V1116 under the same fermentation conditions. Therefore, the genes identified in this thesis would now be evaluated in the most relevant industrial strain background.

Evaluation of the 35 candidate genes was performed in static fermentations in small scale tubes containing 100mL of YP + 33% (w/v) glucose at 25°C. The results of the screening of each overexpressed candidate gene are shown in appendix 3. Because there was some variation in the absolute ethanol concentration achieved by the strains in different fermentation batches, ethanol concentrations are represented as a relative percentage of the reference strain V1116 with the empty plasmid (V1116-pVV214). Confirmation of the strains with promising results in the first screening resulted in five strains with higher ethanol concentration at the end of the fermentation compared to the control strain V1116-pVV214 (Fig. 25A). These were the strains with overexpression of *POX1, THI73, SNQ2, CYB5,* and *COX8. POX1* encodes an acyl-CoA oxidase located in the peroxisomes that plays a critical role in the β-oxidation pathway (Hiltunen et al., 2003); *THI73* encodes a putative plasma membrane permease; *SNQ2* encodes a multidrug pump of the ABC transporter family (Ernst et al., 2005); *CYB5* encodes Cytochrome b5 that is involved in the sterol and lipid biosynthesis pathway (Lamb et al., 1999); and *COX8* encodes subunit VIII of the cytochrome C oxidase complex part of the electron transport chain involved in cellular respiration (Cooper et al., 1991). Those five target genes expressed under the strong *PGK* promoter and the *CYC1* terminator were selected for integration in the genome of V1116 mediated by the Phi-C31 integrase system. The integration was accomplished by replacing one allele of the target gene with the

overexpression cassette. This resulted in a strain with one original allele and the overexpression cassette with the Seg5-derived allele. However, the final ethanol concentration obtained with the constructed strains in VHG fermentation were not affected, or even lowered in case of the *THI73* gene*,* when compared with the reference strain. The first could be explained by the copy number difference between integrative and multi-copy plasmid expression of the overexpression cassettes. However, the second is more difficult to understand (Fig. 25B).

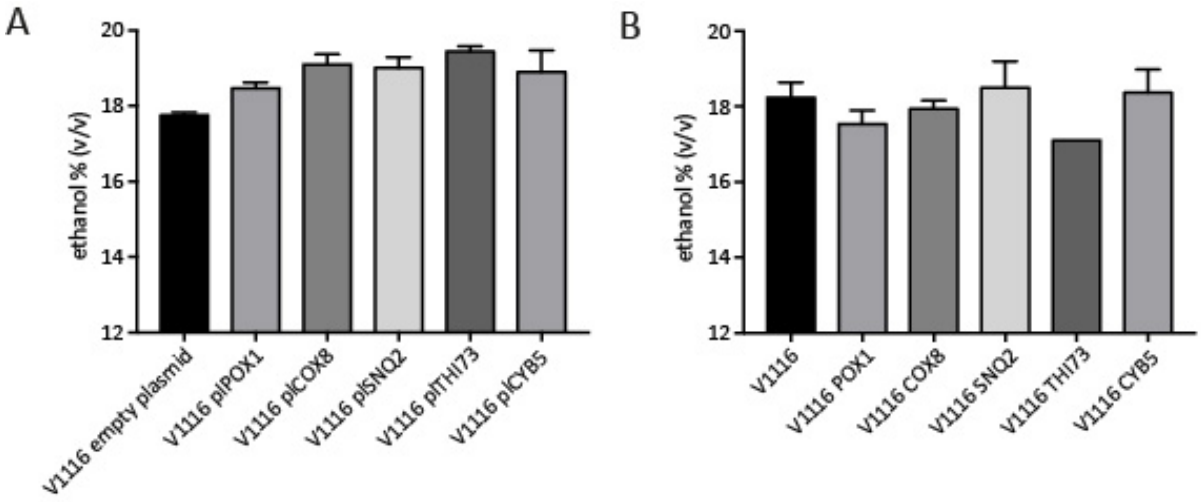


Figure 25: Determination of final ethanol concentration of overexpressed target genes identified by pooled segregant expression analysis. (A) Genes overexpressed on the multi-copy plasmid pVV214 in strain V1116 and the same strain with the empty plasmid as control. (B) Genes integrated in the genome of V1116 and strain V1116 as control.

3 Discussion

S. cerevisiae cells encounter two major stresses during very high gravity fermentation namely high glucose concentrations at the beginning of the fermentation and high ethanol concentrations at the end of the fermentation. Both stressors are known to cause stuck and sluggish fermentations, which lead to reduced productivity of the bioethanol plant. Increased performance of yeast strains during ethanol fermentation can help to increase the productivity and economic viability of the bioethanol industry.

In this study, we performed detailed analysis of QTL9 on chromosome X and QTL17 on chromosome XV and identified two genes *PTK2* and *SHE4* linked to maximal ethanol accumulation capacity, of which the causative gene *SHE4* of QTL17 is also linked to ethanol tolerance of cell proliferation. We have identified the *PTK2* allele derived from the superior haploid parent strain Seg5 by RHA as a superior allele for maximal ethanol accumulation capacity in VHG fermentations. *PTK2* has previously been associated with ethanol tolerance. Deletion of *PTK2* results in a reduced growth rate in liquid medium containing 8% (v/v) ethanol (Yoshikawa et al., 2009). Moreover, Alexandre and coworkers (2001) observed that *PTK2* is up-regulated during short-term ethanol shock although they did not observe any induction of the H^+-ATPase gene (*PMA1*). Furthermore, homozygous deletion of *PTK2* in BY4743 leads to incomplete (stuck) fermentation in high-sugar medium, pointing to *PTK2* as one of the 93 fermentation essential genes or the 'fermentome', a dataset of genes that modulate fermentation (Walker et al., 2014). The finding of *PTK2* as a causative allele involved in maximal ethanol accumulation capacity is consistent with the observation that regulation of H^+-ATPase activity is important during ethanol stress. H^+-ATPase maintains the electrochemical proton gradient, which is important during ethanol stress both for pH homeostasis and ionic homeostasis (Madeira et al., 2010; Walker and Maynard, 1997). *PTK2* encodes a protein kinase, which activates the plasma membrane H^+-ATPase Pma1 by phosphorylating serine residues in the C-terminal part of the protein (Eraso et al., 2006). Ethanol has been known to increase membrane permeability and decrease intracellular pH, resulting in the disruption of the ionic gradients when ethanol accumulates during fermentation. Thereby, the cell requires increasing amounts of energy to re-establish the electrochemical proton gradient (Lam et al., 2014). Lee and co-workers (2017) found that

overexpression of *PMA1* enhanced tolerance to weak acids, reactive oxygen species and ethanol probably due to strengthening the physiology of the yeast cell by establishing more robust proton efflux, lower membrane permeability and less internal hydrogen peroxide production. Several studies have been performed to improve fermentation performance or alcohol tolerance by overexpression of *PMA1*. However, from our study Ptk2*,* which is an activator of Pma1, also appears to be an interesting candidate to engineer industrial yeast strains for improved performance in high gravity fermentations.

Moreover, we have identified a QTL linked to the inferior parent BY4742. Four genes of that QTL, *SHE4*, *PEP12*, *CKB2* and *WHI2,* are known to decrease ethanol tolerance when evaluated in a single gene knockout assay for ethanol tolerance (Auesukaree et al., 2009; Teixeira et al., 2009; Yoshikawa et al., 2009). *SHE4* was identified by RHA as causative for maximal ethanol accumulation capacity as well as for high ethanol tolerance. The superior parent has a *SHE4* allele with a negative effect on ethanol tolerance and/or maximal ethanol accumulation. This may sound surprising; however, it is not unusual (Swinnen *et al*., 2012; Yand *et al*., 2013). We have studied two strains with a clear difference in the trait under investigation. Therefore, it is possible that the inferior strain also contains important positive alleles for the trait but when expressed in that specific background, it does not result in a better phenotype. The She4 protein contains a UCS (UNC-45/CRO1/She4p) domain and is part of the UCS family of proteins, which are chaperones that are specific for folding, assembly and function of myosin. The UCS proteins are myosin-specific chaperones and co-chaperones for Hsp90. In fungi, they are involved in the polarisation of the actin cytoskeleton and asymmetric mRNA localisation (Ni and Odunuga, 2015). *SHE4* has previously been associated with ethanol tolerance in three independent studies screening the single gene-knock out collection for tolerance to different concentrations of ethanol (6-8-10% (v/v)) (Auesukaree et al., 2009; Dudley et al., 2005; Teixeira et al., 2009). Other genes related to actin cytoskeleton organization were also found to be overexpressed in an industrial ethanol tolerant strain CECT10094 compared to a less tolerant strain during the late ethanol stress response (Navarro-Tapia et al., 2016). Kubota and co-workers (2004) also found that the spatial F-actin cytoskeleton is transiently disrupted by addition of ethanol. The actin cytoskeleton is a complex dynamic network of polymers that is essential for the survival of most eukaryotic cells. It plays an important role is many cellular processes like cell shape maintenance, polarity, cell division, endocytosis and vesicular

trafficking (Mishra et al., 2014). There are many indications that a properly functioning actin cytoskeleton network is important to maintain cell viability during ethanol stress.

Global gene expression analysis can also provide information about genes involved in ethanol tolerance or in the mechanism underlying the yeast response to ethanol stress. In the past, many studies have focussed on laboratory strains with moderate ethanol tolerance and their response to short-term ethanol stress in order to understand the mechanisms of ethanol tolerance (Alexandre et al., 2001; Chandler et al., 2004; Fujita et al., 2006). We have performed pooled-segregant gene expression analysis at a specific fermentation time-point in order to identify differently expressed target genes between the superior and inferior parent that could be used to engineer yeast strains for improving maximal ethanol accumulation capacity. Combination of micro-array gene expression analysis and RNA sequencing revealed 37 genes overexpressed in the pool of segregants and the superior parent Seg5, in comparison to BY710. Evaluation of 35 different candidate genes expressed from the multi-copy vector pVV214 in the V1116 wine strain in very gravity fermentation, resulted in five target genes supporting an improvement in maximal ethanol accumulation. Surprisingly, integration of the overexpression cassettes of those five target genes in the genome of V1116 did not result in improvement of maximal ethanol accumulation. This indicates that copy number differences between the integrated overexpression cassette in the genome and the multi-copy plasmid could be the reason for the absence of any improvement in maximal ethanol accumulation. However, combination of the QTL mapping data and gene expression analysis data holds great potential for unravelling the molecular pathways that link driver and target genes underlying complex traits (Ambroset et al., 2011).

In conclusion, we have identified two more causative genes supporting superior maximal ethanol accumulation capacity, which expands our understanding of the molecular mechanisms involved in general ethanol tolerance as well as in maximal ethanol accumulation capacity in VHG fermentations. Precision engineering of these genes might allow us to further improve or design new industrial yeast strains with better fermentation performance under very high gravity conditions. Unfortunately, we could not yet improve maximal ethanol accumulation in the strain V1116 wine strain by overexpression of the most promising target genes upon stable integration in the genome. However, further engineering in order to obtain

higher expression of the integrated constructs has a clear potential to provide positive results for improving maximal ethanol accumulation in a stable manner.

CHAPTER IV

Improvement of ethanol tolerance and maximal ethanol accumulation capacity in ER18

1 Introduction

Complex polygenic traits, like ethanol tolerance and maximal ethanol accumulation capacity, are determined by multiple genes. In previous chapters, we have identified different superior alleles for both traits. With pooled-segregant whole-genome sequence analysis methodology, we have identified the superior alleles $MKT1_{VR1\text{-}5B}$, $APJ1_{VR1\text{-}5B}$ and $VPS70_{VR1\text{-}5B}$ for high ethanol tolerance (Chapter II). Moreover, we have found $ADE1_{Seg5}$, $KIN3_{Seg5}$, $PTK2_{Seg5}$ and $SHE4_{BY4742}$ as the superior alleles for maximal ethanol accumulation capacity (Chapter III). An overview of the specific mutations can be found in figure 28 (see below).

Engineering yeast strains for complex traits is still a time-consuming process especially when multiple successive gene manipulations are required. Limitations of dominant, selectable markers still present a bottleneck for making knock-outs of multiple genes in laboratory or industrial yeast strains. Additionally, excision of selectable markers can be done by homologous recombination (HR)-mediated counter selection, or by the use of recombinases. Cre and Flp recombinases recognise two loxP and FRT sites, respectively, leaving a copy of that repeat sequence in the genome, which could lead to genome instability by unwanted chromosome rearrangements (Jakociunas et al., 2015). An alternative is Phi-C31 integrase, which mediates sequence-specific recombination between two largely different attachment sites, called attB and attP, that share a 3 bp central region where the crossover occurs, and gives as a result attR and/or attL sites. The integration is specific and unidirectional, hence, the remaining scar left after recombination is completely inactive (Groth et al., 2000; Kuhstoss and Rao, 1991). Moreover, it is also possible to excise 'scar-less' markers via the delitto perfetto method or I-SceI-induced double stranded breaks, however, it remains a time-consuming process (Solis-Escalante et al., 2014; Storici et al., 2001). Marker-free modifications using double-stranded DNA breaks (DSBs) for site-directed genome editing, such as meganucleases, zinc finger nucleases (ZFNs) (Carroll, 2011) and transcription activator-like effector nucleases (TALENs) (Mussolino et al., 2011), are very time-consuming and expensive alternative methods, because each time a new ZFN or TALEN protein has to be designed (Stovicek et al., 2015).

Recently, another simple and efficient tool for marker-free genome editing has been developed originating from the bacterial adaptive immune system, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - Cas9 technology (Doudna and Charpentier, 2014). The most commonly used CRISPR-associated protein Cas9 is an endonuclease from *Streptococcus pyogenes*, which introduces a DSB at a specific genomic site (Laughery et al., 2015). Cas9 is guided to the DNA target sequence by a single guide RNA (sgRNA). The sgRNA consists of 20 bp sequence with two features, a sequence at 5' side that determines the DNA target site and a duplex RNA structure at the 3' side that binds to Cas9. Correct DNA target recognition requires both sgRNA base pairing and the presence of a protospacer adjacent motif (PAM) 5'-NGG-3' sequence adjacent to the DNA target sequence (Jinek et al., 2012). By redesigning the 5' side of the sgRNA molecule the system can be used to target any desired sequence when there is a PAM site close to the target genomic site (Cong et al., 2013).

In this last chapter, after evaluating different industrial yeast strains for maximal ethanol accumulation capacity and ethanol tolerance of cell proliferation on solid medium, we have chosen the haploid segregant ER18, with low ethanol production and ethanol tolerance, to further improve both phenotypes. The different superior alleles involved in ethanol tolerance and maximal ethanol accumulation capacity, which were identified by pooled-segregant whole genome sequence analysis, were engineered in the haploid yeast strain ER18 using CRISPR-Cas9 technology. We successfully improved ER18 for ethanol tolerance and maximal ethanol accumulation capacity by introducing a specific combination of superior alleles.

2 Results

2.1 Characterisation of industrial yeast strains for maximal ethanol accumulation and ethanol tolerance.

Eighteen yeast strains were characterised for their ethanol tolerance on YPDEtOH plates by spot assays and for their maximal ethanol accumulation capacity in VHG fermentations. Those 18 yeast strains comprised industrial strains for 1st and 2nd generation bioethanol production (1G and 2G), for wine and saké production, the laboratory strain S288c, and haploid segregants of the 1G industrial strain Ethanol Red (ER) (Table1). To determine the ethanol tolerance of a strain, we scored the growth of each strain on YPD plates with different concentrations of ethanol ranging from 8% to 18% (v/v). We defined ethanol tolerance as the maximum ethanol concentration at which its growth was still visually the same as its growth on the control YPD plate without ethanol and an example is shown in Fig. 26. Semi-static fermentations were performed in small-scale tubes containing 100 mL YP + 33% (w/v) glucose at 32°C with an inoculum size of OD_{600} 4. Maximal ethanol concentration was determined by HPLC at the end of each fermentation (Fig. 27). A summary of the results for ethanol tolerance and ethanol production capacity for the different strains is shown in Table 7.

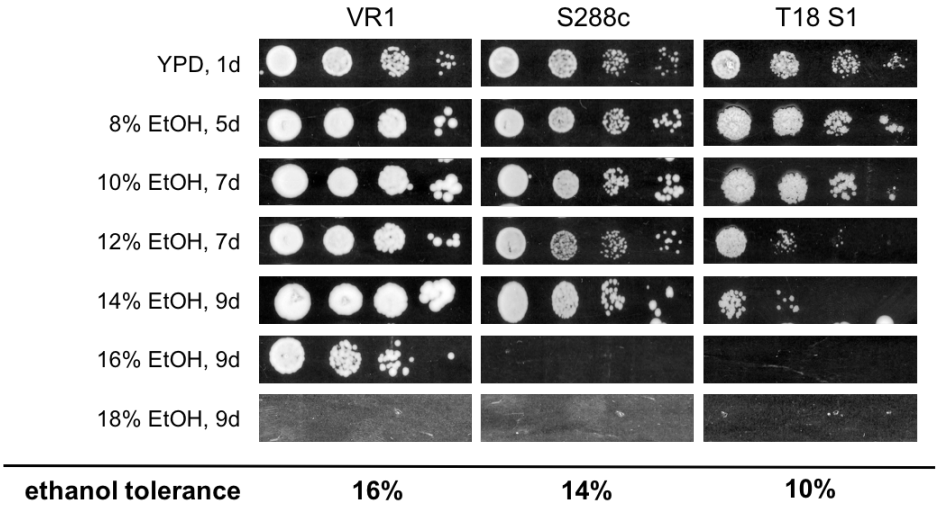


Figure 26: Determination of ethanol tolerance of different yeast strains. Growth of a 10-fold dilution range of 3 yeast strains was determined on YPD plates containing different ethanol concentrations, from 8 to 18% (v/v). Ethanol tolerance was defined as the ethanol concentration at which growth was still the same as that on the control YPD plate.

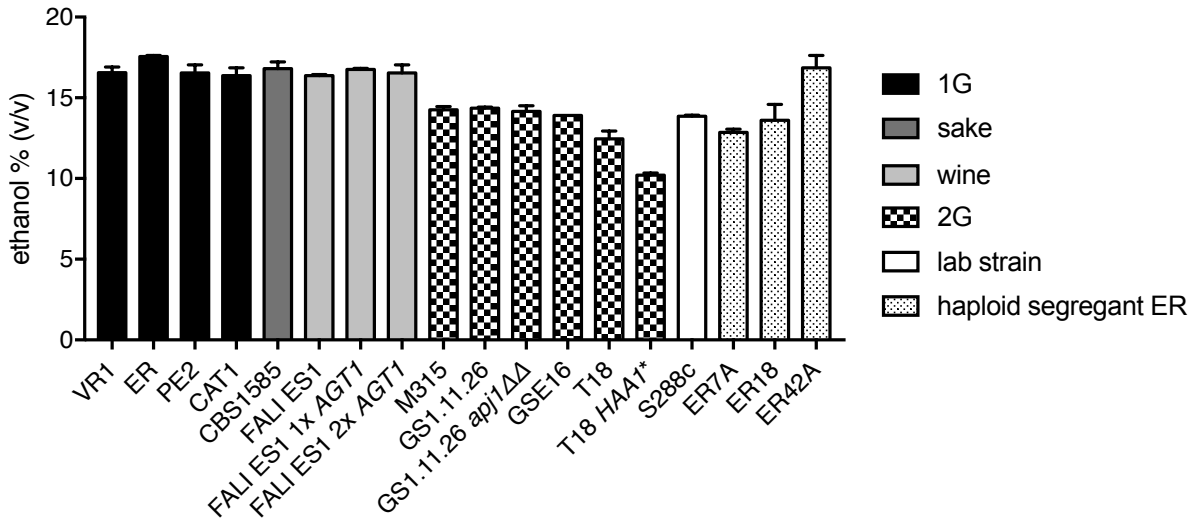


Figure 27: Final ethanol concentration accumulated by 19 yeast strains. VHG fermentations were performed at 32°C in 100 mL of YP + 33% (w/v) glucose. The inoculum size was OD_{600} 4 and fermentations were semi-static, only stirred during the first 4 h at 120 rpm. Yeast strains are grouped according to origin (see legend).

Table 7: Characterization of 18 yeast strains for maximal ethanol accumulation capacity in VHG fermentations and their ethanol tolerance of cell proliferation on YPDEtOH plates.

Yeast strain	**Origin**	**Ethanol tolerance % (v/v)**	**Ethanol production % (v/v)**
VR1	1G (2n)	16	16.6 ± 0.4
ER	1G (2n)	14	17.6 ± 0.1
PE2	1G (2n)	nd.	16.6 ± 0.5
CAT1	1G (2n)	nd.	16.3 ± 0.5
CBS1585	saké (2n)	18	16.8 ± 0.4
FALI ES1	wine (2n)	14	16.4 ± 0.1
FALI ES1 1X *AGT1*	wine (2n)	14	16.7 ± 0.1
FALI ES1 2x *AGT1*	wine (2n)	14	16.5 ± 0.5
M315	2G (2n)	12	14.3 ± 0.2
GS1.11-26	2G (2n)	12	14.3 ± 0.1
GS1.11-26 *apj1*ΔΔ	2G (2n)	12	14.2 ± 0.3
GSE16	2G (2n)	nd.	13.9 ± 0.0
T18	2G (2n)	10	12.5 ± 0.5
T18 *HAA1**	2G (2n)	<10	10.2 ± 0.1
S288c	Laboratory strain (2n)	14	13.8 ± 0.1
ER7A	segregant ER (n)	12	12.9 ± 0.2
ER18	segregant ER (n)	<10	13.6 ± 1.0
ER42A	segregant ER (n)	18	16.9 ± 0.8

1G, 1st generation bioethanol production; 2G, 2nd generation bioethanol production; ER, Ethanol Red; n, ploidy.

The growth assays showed that the diploid industrial strains for 1G bioethanol, wine and saké production had a high ethanol tolerance, ranking from 14% to 18%. The laboratory strain S288c could also grow in the presence of 14% (v/v) ethanol. However, the strains engineered in our laboratory for 2G bioethanol production were less tolerant (10 to 12% (v/v) ethanol). The 3 segregants randomly selected from the industrial strain ER showed a high variability, with the segregant ER42A being the most tolerant (18 % (v/v) ethanol tolerance), and even more tolerant than its parent strain.

Ethanol tolerance and maximal ethanol accumulation capacity are two distinguishable traits that however can share some underlying genetic elements. In our study, the strains showed a similar behaviour for both traits. For maximal ethanol accumulation capacity in VHG fermentations the industrial yeast strains used for 1G bioethanol and wine/sake production accumulated more than 16% (v/v) ethanol, which was expected from previous reports on high ethanol production (Martins Pais, 2012). The strains for 2G bioethanol production produced on average an ethanol concentration of 14% (v/v), which was the lowest production level, and they were also less tolerant to ethanol for proliferation on solid nutrient medium (T18 and T18 *HAA1*,* accumulating 12.5% ± 0.5 and 10.2% ± 0.1 (v/v), respectively).

Among the strains tested, those engineered for 2G bioethanol production lost their ability to produce high ethanol concentrations in rich fermentation media with 33% glucose, although they were selected for optimal performance in xylose fermentation and inhibitor tolerance in lignocellulose hydrolysate. Moreover, the segregant ER18 was very sensitive to ethanol and accumulated 4% (v/v) less ethanol than its parent strain. This strain is haploid and was therefore chosen to further improve its maximal ethanol accumulation capacity and ethanol tolerance of cell proliferation.

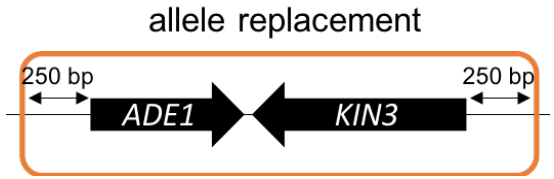
2.2 Engineering ER18 for higher ethanol tolerance and maximal ethanol accumulation capacity using CRISPR-Cas9 technology

Improving maximal ethanol accumulation of industrial yeast strains is of great economic importance for the bioethanol production industry. As explained above (section 2.1), we selected the haploid segregant ER18 of the industrial yeast strain Ethanol Red to improve its maximal ethanol accumulation capacity and ethanol tolerance. To achieve those goals, the ER18 strain was engineered with the superior alleles $MKT1_{VR1\text{-}5B}$, $APJ1_{VR1\text{-}5B}$, $VPS70_{VR1\text{-}5B}$, $ADE1_{Seg5}$ and $KIN3_{Seg5}$ (see chapter II; Pais et al., 2013; Swinnen et al., 2015; Swinnen et al., 2012). The superior alleles were tested alone or combined, in order to find the best combination for further improvement of our in-house 2G bioethanol production strains (T18 and T18 *HAA1**), which showed only low ethanol tolerance and accumulation capacity.

The first step was to compare the sequence of the superior alleles with those of the alleles present in the ER18 strain, which showed that all the sequences were different (Fig. 28). Next, the ORFs of the ER18 alleles were replaced by the corresponding superior alleles, except for *ADE1* and *KIN3.* Both alleles are in close proximity to each other (100bp). No nonsynonymous mutation could be found between Seg5 and ER18 for the *ADE1* and *KIN3* sequences, but 1 SNP in the promoter of *ADE1*, several SNPs in the promoter of *KIN3* and four synonymous mutations in the ORF of *KIN3* were present. Therefore, both genes were replaced with part of the promoter sequence (250 bp) (Fig. 28A), assuring that the unique SNPs described by Pais and co-workers (2013) were included.

Allele replacements were performed by CRISPR-Cas9 technology. First, the endogenous allele was knocked-out by replacing the ORF with an antibiotic resistance marker flanked with a unique gRNA recognition site, named G2. The KO and Cas9-expressing ER18 was co-transformed with the G2-gRNA plasmid and the donor DNA. The donor DNA was a linear DNA fragment of the superior allele amplified from the specific yeast strain (Seg5 or VR1-5B) and used to repair the lethal double strand break caused by Cas9. The resulting transformants were analysed by allele-specific PCR.

A



	Prom *ADE1*	Prom *KIN3*							ORF *KIN3*			
Position	**-148**	**-428**	**-412**	**-321**	**-317**	**-316**	**-272**	**-261**	**-244**	**315**	**759**	**852**	**1040**
S288c	C	T	G	T	-	-	T	A	T	T	T	G	C
Seg5	T	A	T	T	T	T	T	T	-	A	T	C	A	T
ER18	C	A	T	C	-	-	C	-	T	C	T	G	C
	allele replacement								allele replacement				

B

	ORF *MKT1*										
position	**89**	**213**	**384**	**543**	**978**	**1050**	**1057***	**1308**	**1314**	**1358**	**1464**
S288C	A	G	G	T	A	G	A	A	T	A	G
VR1-5B	G	A	A	C	G	G	A	G	T	G	G
ER18	G	G	A	T	G	A	G	G	C	G	A
Amino acid change in ER18 vs VR1-5B							**K(353)E**				

	ORF *MKT1*									
position	**1496***	**1572**	**1590**	**1710**	**1731**	**1782**	**1800***	**1828***	**2001**	**2040**
S288C	C	A	C	C	A	G	C	A	T	T
VR1-5B	C	G	T	T	G	A	A	C	C	C
ER18	A	A	C	C	A	G	C	A	T	T
Amino acid change in ER18 vs VR1-5B	**T(499)N**						**L(600)F**	**R(610)S**		

C

	ORF *VPS70*										
position	**25***	**129**	**260***	**596**	**678**	**757***	**762**	**832**	**980***	**1149**	**1260**
S288C	A	C	A	C	A	A	T	T	G	T	C
VR1-5B	G	T	A	T	A	A	C	C	A	T	T
ER18	A	C	G	T	G	G	C	C	G	C	C
Amino acid change in ER18 vs VR1-5B	**E(9)K**		**K(87)R**			**S(253)G**			**K(327)R**		

	ORF *VPS70*										
position	**1284**	**1383**	**1420**	**1425**	**1581**	**1623**	**1656**	**1729**	**1868***	**2123**	**2236**
S288C	G	C	T	T	G	G	T	T	A	G	T
VR1-5B	G	T	C	T	G	C	T	T	A	A	T
ER18	A	C	T	G	A	G	C	C	G	A	C
Amino acid change in ER18 vs VR1-5B									**N(623)S**		

D

	ORF *APJ1*						
Position	**12**	**144**	**186**	**367***	**435**	**455***	**1188**
S288c	C	T	T	G	C	G	G
VR1-5B	T	C	C	G	T	G	A
ER18	T	C	C	A	T	A	A
Amino acid change in ER18 vs VR1-5B				**A(123)T**		**S(152)N**	

Figure 28: Occurrence of the SNPs and amino acid changes in the different causative genes (ADE1- KIN3 (A), MKT1 (B), VPS70 (C) and APJ1 (D)) compared to the sequence of S288c. SNPs are colored grey. Amino acid changes between the superior allele and the allele of ER18 are shown in the bottom line in bold face type.

2.2.1.1 Single gene replacement of superior alleles in ER18

As explained above, all superior alleles were assessed individually, except for *ADE1* and *KIN3*. In the case of *APJ1*, both the deletion and the superior allele were tested. The growth of the different ER18 mutant strains was compared on YPD medium containing different ethanol concentrations (10% to 14% (v/v)) (Fig. 29). The results revealed that the superior alleles $ADE1_{Seg5}$ and $KIN3_{Seg5}$ and $MKT1_{VR1\text{-}5B}$ clearly improve the ethanol tolerance of ER18 from 10 to almost 14% (v/v) ethanol. Also, allele replacement of $VPS70_{VR1\text{-}5B}$ resulted in improvement of ethanol tolerance although there was less growth on YPD medium containing 14% (v/v) ethanol. Allele replacement with $APJ1_{VR1\text{-}5B}$ or *APJ1* deletion in ER18 did not contribute to any growth improvement in the presence of ethanol compared to the control strain. Therefore, the superior alleles $ADE1_{Seg5}$, $KIN3_{Seg5}$ and $MKT1_{VR1\text{-}5B}$ conferred the highest contribution in increasing ethanol tolerance in ER18 followed by *VPS70*, *apj1Δ* and the *APJ1* allele from the strain VR1-5B.

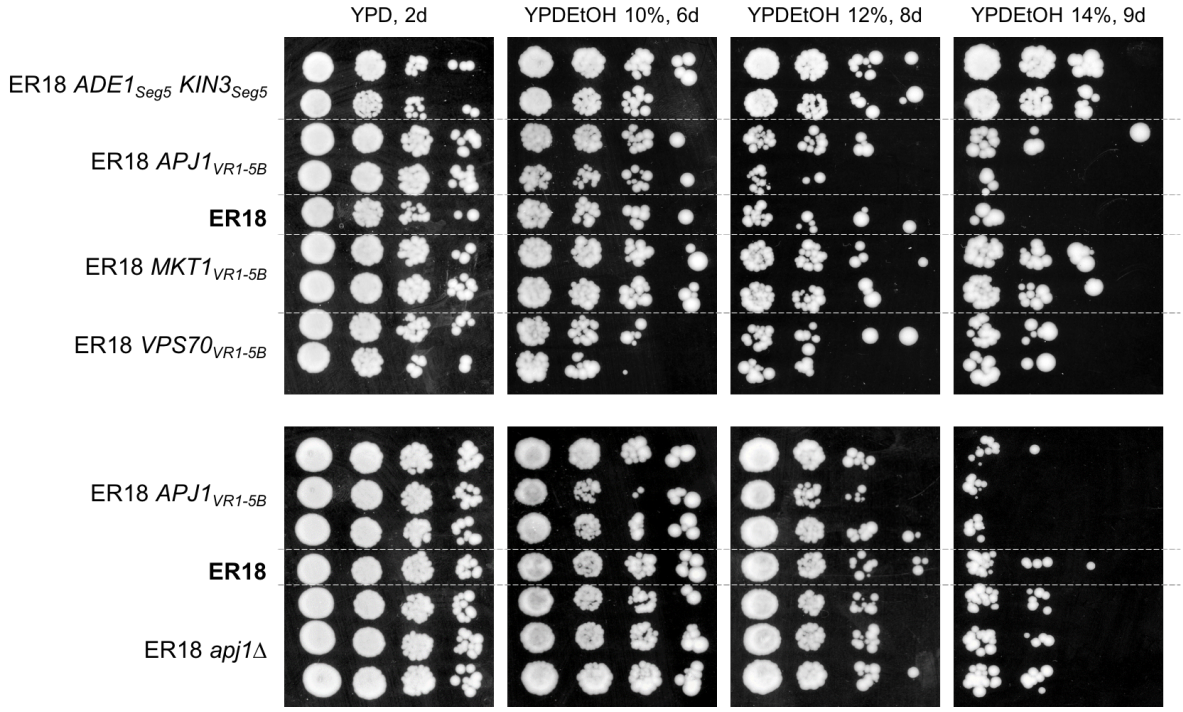


Figure 29: Ethanol tolerance of cell proliferation in mutant ER18 strains, expressing superior alleles previously identified for ethanol tolerance and ethanol accumulation capacity. Growth of tenfold dilutions of these strains were scored on YPD plates in the absence and in the presence of different ethanol concentrations (10 to 14% (v/v)) and at the times indicated.

The effect of single gene replacement in ER18 on ethanol production was evaluated in VHG fermentations. Semi-static fermentations were performed in small-scale tubes containing 100 mL of YP medium with 33% (w/v) glucose. The final ethanol concentration accumulated by these strains is shown in figure 30. In contrast to the results obtained for ethanol tolerance of cell proliferation, the ER mutants *apj1Δ* and $VPS70_{VR1\text{-}5B}$ showed a significant improvement of ethanol production during VHG fermentation. The $VPS70_{VR1\text{-}5B}$ allele replacement caused an improvement from 11.2% ± 0.1 to 13.9% ± 0.8 (v/v) ethanol. Deletion of *APJ1* in ER18 increased ethanol production from 11.2% ± 0.1 to 14.4% ± 0.1 (v/v) under these fermentation conditions. On the other hand, the mutants expressing the superior alleles $ADE1_{Seg5}$, $KIN3_{Seg5}$ and $MKT1_{VR1\text{-}5B}$ produced the same ethanol level as the ER18 stain.

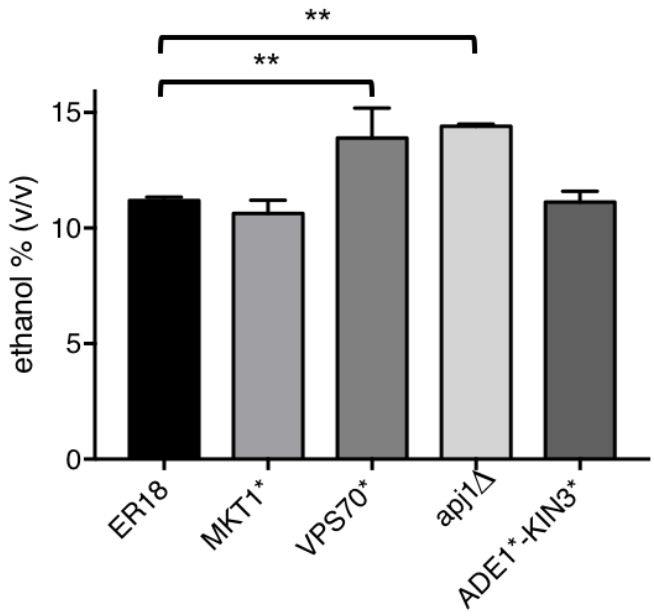


Figure 30: Final ethanol concentration produced by mutant ER18 strains, expressing superior alleles previously identified for ethanol tolerance and ethanol accumulation capacity. VHG fermentations were performed at 32°C in 100 mL YP + 33% (w/v) glucose. The inoculum size was OD_{600} 4. The fermentations were semi static, only being stirred during the first 4 h at 120 rpm. Each fermentation was done in triplicate. The results were analysed with one-way ANOVA (comparison to control strain ER18, Dunnett's multiple comparison). (Statistical significance is indicated by p-values (**) between 0.01 and 0.001).

2.2.1.2 Multiple gene replacement of superior alleles in ER18

Single allele replacement could improve ER18 for ethanol tolerance of cell proliferation or maximal ethanol production. Therefore, combinations of the superior alleles engineered into

the ER18 haploid strain were evaluated in order to find the ideal combination to maximize ethanol tolerance of cell proliferation and/or maximal ethanol production. The first comparison between different allele combinations was done by using the strain ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ as the base strain and introducing other superior alleles separately (Fig. 31). In this way, we could see which one had an additional effect. When ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ was combined with *VPS70*$_{VR1\text{-}5B}$ or *MKT1*$_{VR1\text{-}5B}$, ethanol tolerance increased from 10 to 14% ethanol and even growth on YPDEtOH 16% was observed. We have to note that the two ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *apj1Δ* strains showed conflicting results. After double checking the genotype of the strains by PCR, the reason for these conflicting results remained unclear. In general, ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ in combination with *APJ1*$_{VR1\text{-}5B}$ or *apj1Δ* decreased ethanol tolerance compared to ER18 (except for one strain), which indicates that *APJ1* has an adverse effect in combination with *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ for ethanol tolerance in ER18. Also, the strain ER18 *VPS70*$_{VR1\text{-}5B}$ was combined with the other superior alleles (Fig. 32). Again, ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *VPS70*$_{VR1\text{-}5B}$ showed the best improvement of ethanol tolerance. Any other combination also contributed to a higher ethanol tolerance, and again the combination with *APJ1*$_{VR1\text{-}5B}$ caused a negative effect for growth on YPDEtOH plates. Moreover, the only combination with *MKT1*$_{VR1\text{-}5B}$ that clearly improved ethanol tolerance for growth on solid nutrient medium was ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *MKT1*$_{VR1\text{-}5B}$ (Fig. 33). When we compared the growth of ER18 *APJ1*$_{VR1\text{-}5B}$ in combination with the other superior alleles, previous results were confirmed. Furthermore, the beneficial effect of the other superior alleles diminished in combination with the *APJ1* derived allele of VR1-5B (Fig. 34).

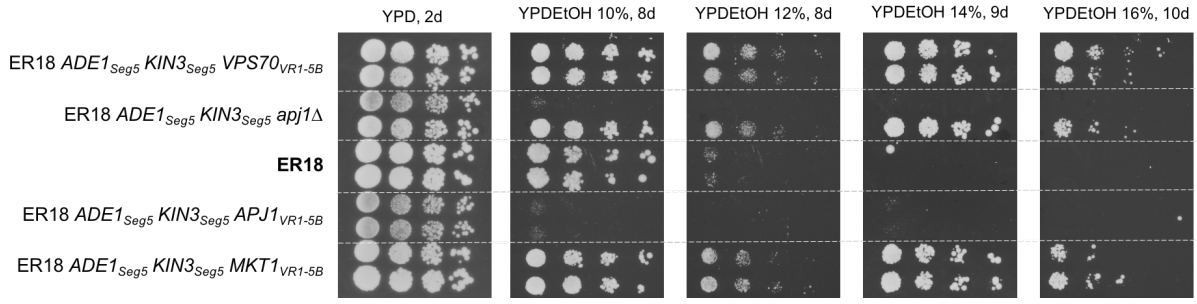


Figure 31: Ethanol tolerance of the ER18 ADE1$_{Seg5}$ *KIN3*$_{Seg5}$ *strain in combination with other superior alleles on media containing moderate to high concentrations of ethanol. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated.*

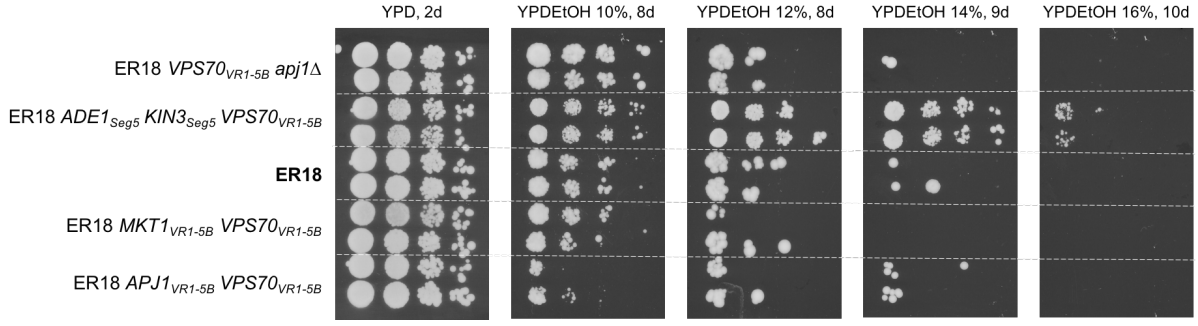


Figure 32: Ethanol tolerance of the ER18 $VPS70_{VR1\text{-}5B}$ strain in combination with other superior alleles on media containing moderate to high concentrations of ethanol. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated.

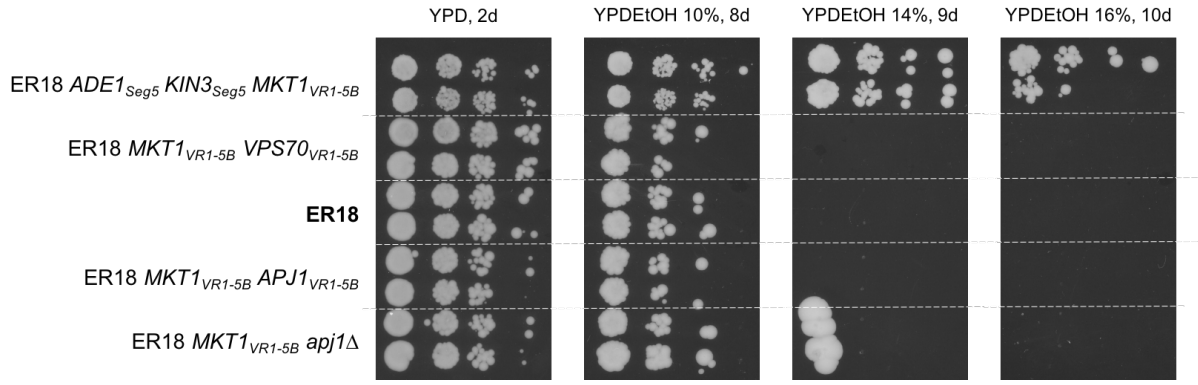


Figure 33: Ethanol tolerance of the ER18 $MKT1_{VR1\text{-}5B}$ strain in combination with other superior alleles on media containing moderate to high concentrations of ethanol. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated.

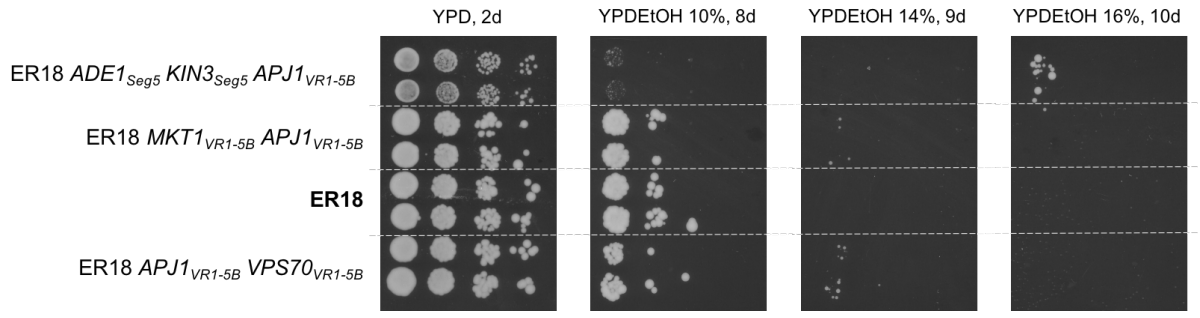


Figure 34: Ethanol tolerance of the ER18 $APJ1_{VR1\text{-}5B}$ strain in combination with other superior alleles on media containing moderate to high concentrations of ethanol. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated.

Figure 35 shows the growth of seven different strains with triple combinations of the superior alleles in the ER18 background on YPD plates with high ethanol concentrations. We observe that combinations with *APJ1*$_{VR1\text{-}5B}$ showed less improvement than combinations with *apj1Δ*. ER18 *APJ1*$_{VR1\text{-}5B}$ *MKT1*$_{VR1\text{-}5B}$ *VPS70*$_{VR1\text{-}5B}$ and ER18 *apj1Δ MKT1*$_{VR1\text{-}5B}$ *VPS70*$_{VR1\text{-}5B}$ showed no improvement for ethanol tolerance. When 3 superior alleles were present together with a deletion of *APJ1,* the negative effect of *apj1Δ* was minimized compared to the double replacement of superior alleles with *apj1Δ*. Overall, we can conclude that ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *MKT1*$_{VR1\text{-}5B}$ *VPS70*$_{VR1\text{-}5B}$ showed the largest improvement of ethanol tolerance, from 10 to 16% (v/v) ethanol. Also, ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *apj1Δ MKT1*$_{VR1\text{-}5B}$ and ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *apj1Δ VPS70*$_{VR1\text{-}5B}$ showed increased ethanol tolerance (Fig. 35).

Finally, we have evaluated the combinations of all the superior alleles introduced in ER18 for growth on YPD plates containing high ethanol concentrations (Fig. 36). The combinations with *apj1Δ* or *APJ1*$_{VR1\text{-}5B}$ caused a clear improvement in ethanol tolerance compared to that of the control strain ER18 (Fig. 36). Unfortunately, it seems that introduction of all the superior alleles does not further improve ethanol tolerance as determined on YPDEtOH plates compared to that of the strain ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *MKT1*$_{VR1\text{-}5B}$ *VPS70*$_{VR1\text{-}5B}$ as shown in figure 35. In general, we have improved ethanol tolerance of the strain ER18 from 10 to 16% (v/v) ethanol by introduction of the superior alleles *ADE1*$_{Seg5}$ *KIN3*$_{Seg5,}$ *MKT1*$_{VR1\text{-}5B}$ and *VPS70*$_{VR1\text{-}5B.}$ Introduction of the superior allele *APJ1*$_{VR1\text{-}5B}$ or *apj1Δ* did not result in any improvement of ethanol tolerance in ER18.

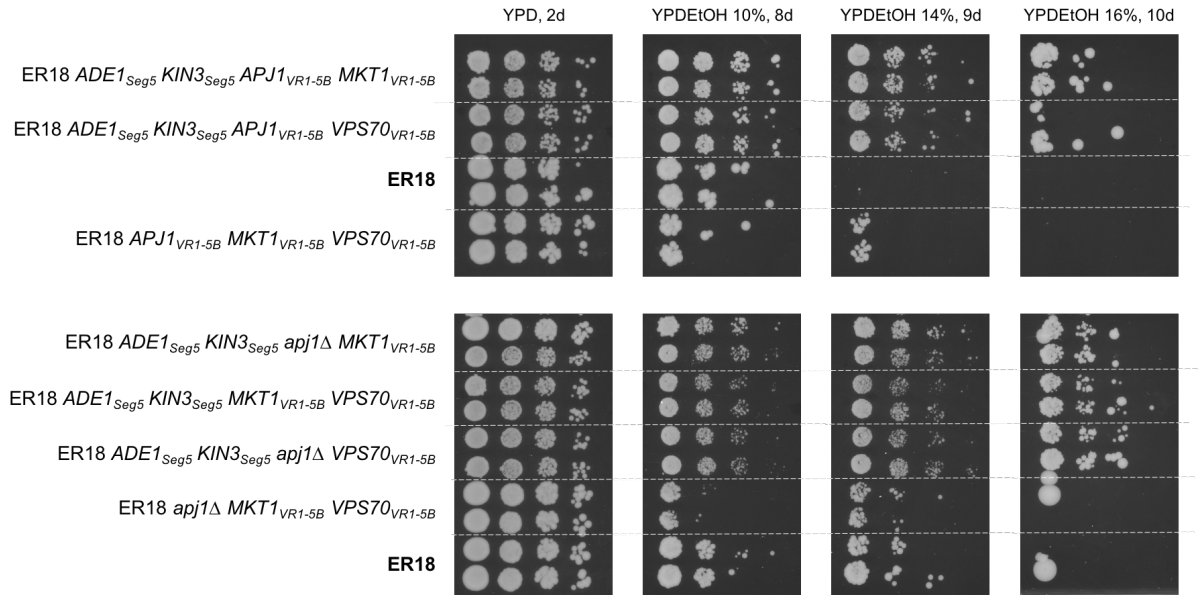


Figure 35: Ethanol tolerance of ER18 containing triple combinations of the superior alleles. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated

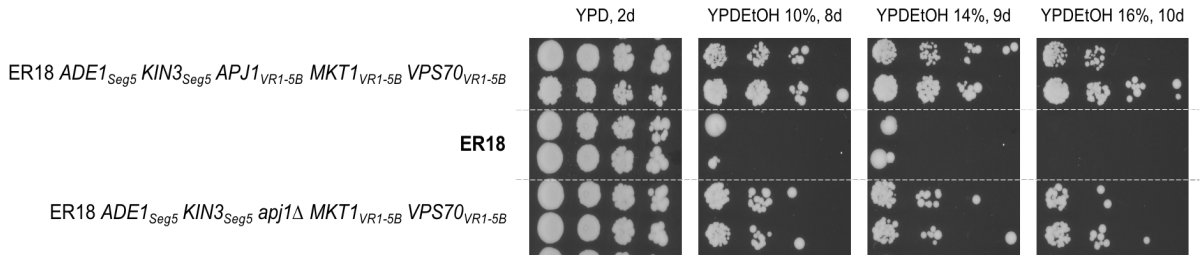


Figure 36: Ethanol tolerance of ER18 containing all superior alleles for higher ethanol tolerance and maximal ethanol accumulation capacity. A 10-fold dilution range of each strain was spotted onto YPD medium containing 10 to 16% (v/v) ethanol and subsequently grown for different periods of time, as indicated.

To investigate the effect on maximal ethanol production of the multiple gene replacement in ER18 with the superior alleles, we have evaluated the constructed strains in VHG fermentations. ER18 *ADE1*$_{Seg5}$ *KIN3*$_{Seg5}$ *VPS70*$_{VR1\text{-}5B}$ produced the highest concentration of ethanol under these conditions (13.8% ± 0.2 (v/v) of ethanol) although not higher than the single allele replacement of *VPS70*$_{VR1\text{-}5B}$ in ER18 (13.9% ± 1.3 (v/v) of ethanol). Other strains with double allele replacement did not produce significantly more ethanol compared to the control strain ER18 (Fig. 37). In contrast to the single allele replacement of *apj1Δ* or *VPS70*$_{VR1\text{-}5B}$ in ER18, both alleles together did not cause a further increase in maximal ethanol production. Triple superior allele replacement in ER18 could not bring about a significant improvement in maximal ethanol production capacity. Therefore, triple allele replacement in ER18 does not bring added value to maximize ethanol production under these conditions. Introduction of all the superior alleles together in ER18 did not result in a significant improvement of maximal ethanol production compared to the wild type strain although visual analysis shows improvement (13.2% ± 0.14 (v/v) of ethanol) (Fig. 37). In agreement with previous results on ethanol tolerance, we can therefore conclude that introduction of the superior alleles *ADE1*$_{Seg5}$*, KIN3*$_{Seg5}$ *and VPS70*$_{VR1\text{-}5B}$ in the ER18 strain could increase the tolerance to high ethanol concentrations of cell proliferation and enhance maximal ethanol production capacity of this strain in VHG fermentations.

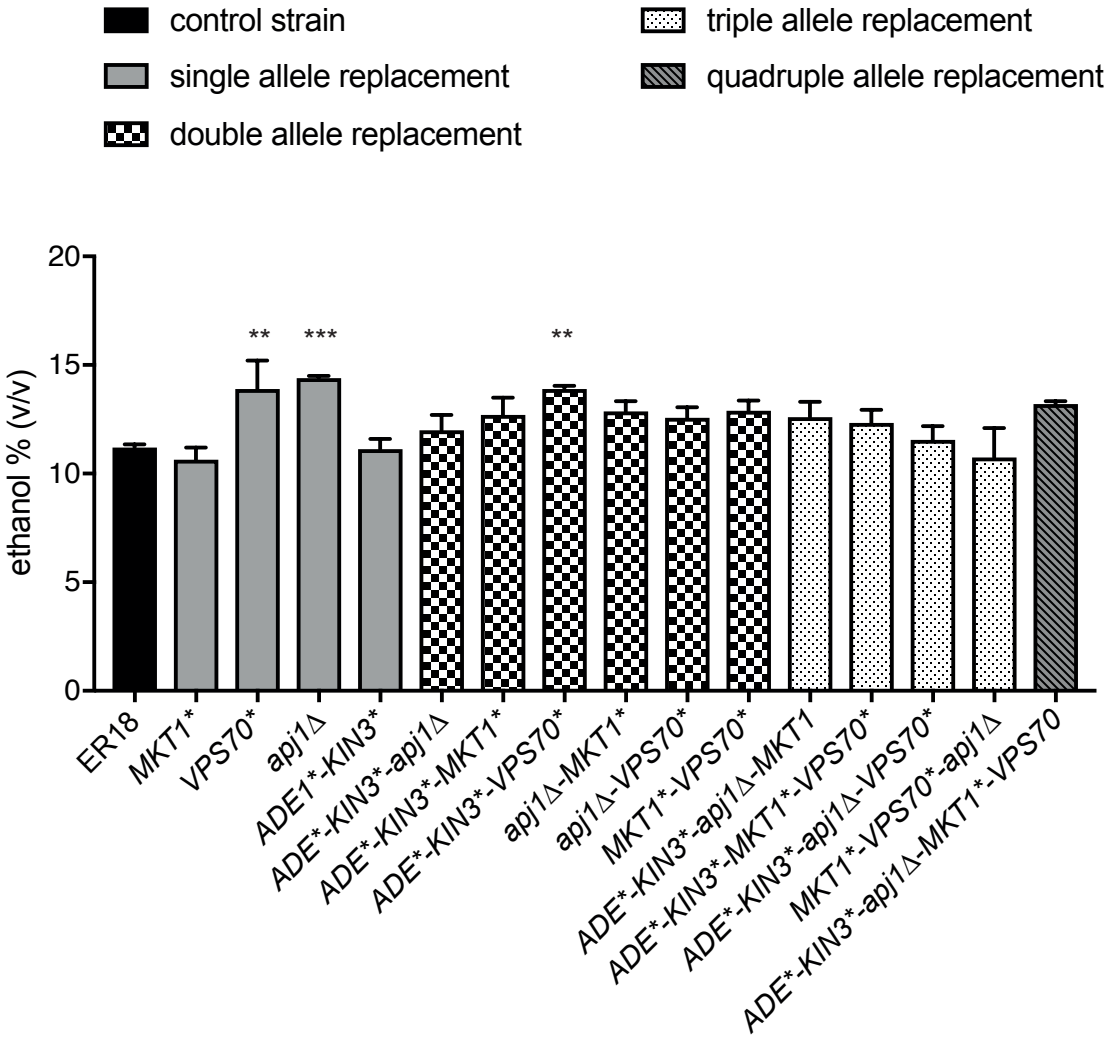


Figure 37: Final ethanol concentration of mutant ER18 strains, expressing superior alleles previously identified for ethanol tolerance and maximal ethanol accumulation. VHG fermentations were performed at 32°C in 100 mL YP + 33% (w/v) glucose. The inoculum size was OD_{600} 4 and fermentations were semi static with stirring at 120 rpm only for the first 4 h. Each fermentation was done in triplicate. The results were analysed with one-way ANOVA (comparison to control strain ER18, Dunnett's multiple comparison). (Statistical significance is indicated by p-values (**) between 0.01 and 0.001, (***) between 0.001 and 0.0001).

3 Discussion

The goal of the present study was to evaluate the applicability of superior alleles identified for their contribution to ethanol tolerance and/or maximal ethanol accumulation in the development of superior industrial yeast strains for bioethanol production. First, the strains were evaluated for both traits, which showed that strains with a low ethanol tolerance of cell proliferation on solid nutrient medium also displayed poor maximal ethanol accumulation in VHG fermentations and vice versa. This contrasts with the study of Pais and co-workers (2013) who tested 68 strains for both traits and found a weak correlation between the two traits. As expected, 1G bioethanol, wine and sake strains showed the best maximal ethanol production, which fits with their industrial application (Katou et al., 2008; Lopes et al., 2016; Walker and Stewart, 2016). On the other hand, the 2G bioethanol production strains, S288c and the haploid segregants of ER displayed the lowest ethanol tolerance/production. Although the 2G bioethanol production strains have in part an ER background, they were engineered for improved xylose fermentation and inhibitor tolerance in hydrolysate by mutagenesis and genome shuffling (Demeke et al., 2013b). Both techniques are promising to improve strains for industrial traits, although there is the danger of losing other important traits by inadvertent introduction of deleterious mutations, which could have happened in these strains. Moreover, some haploid segregants of ER (ER7A and ER18) behaved rather atypical, displaying opposite characteristics compared to their parent industrial strain, which are low ethanol tolerance of cell proliferation on YPD plates with high concentrations of ethanol and low maximal ethanol production in VHG fermentations. Therefore, these segregants must have inherited a combination of alleles that confers their weak performance. We decided to choose the ER18 segregant as parent strain for evaluation of the usefulness of the causative alleles for improvement of the two phenotypes. Improvement of this segregant of the ER strain, could give an indication of the most interesting superior alleles to improve our in-house 2G bioethanol production strains (T18 and T18 *HAA1**) which have in part an ER-background.

Hence, the different superior alleles, $ADE1_{Seg5}$, $KIN3_{Seg5}$, $APJ1_{VR1\text{-}5B}$, $MKT1_{VR1\text{-}5B}$ and $VPS70_{VR1\text{-}5B}$, identified by pooled-segregant whole-genome sequence analysis for high ethanol tolerance and maximum ethanol accumulation in previous projects (Pais et al., 2013; Swinnen

et al., 2012) were evaluated individually and in combination in the ER18 strain background. Allele replacement of the inferior alleles by the superior alleles in the ER18 strain was performed using CRISPR-Cas9 technology, a powerful new tool for genome editing. This methodology has proven to be easily applicable and extremely useful (Stovicek et al., 2015). However, there are still some concerns like off-target effects (Zhang et al., 2015) and the many cloning steps needed to generate multiple sgRNAs for Cas9 multiplexing (Ryan and Cate, 2014). In this study, we made use of antibiotic markers flanked by a unique gRNA sequence (G2) in order to remove markers in a scar-free manner. In this way, we could use the CRISPR-Cas9 technology for multiple allele replacement without constructing strain-specific gRNA for each gene. Although this approach still results in a two-step methodology, a strong advantage is that it is not strain dependent. Nevertheless, this two-step methodology is not applicable for allele replacement of essential genes. Direct replacement of essential genes in a haploid background is possible by using CRISPR-Cas9 technology with two guide RNAs, one targeting a sequence upstream of the allele and the other targeting a sequence downstream of the allele of interest (Trindade de Carvalho et al., 2017).

In this work, we have constructed different ER18 strains with single, double, and quadruple superior alleles identified for high ethanol tolerance and maximal ethanol accumulation. To the best of our knowledge, this was the first time that different causative genes identified by pooled-segregant whole-genome sequence analysis were combined into a single strain in order to further improve the trait of interest. In the past, other approaches like single gene manipulation (deletion or overexpression) have been applied to improve yeast strains for ethanol tolerance (Divate et al., 2016; Krause et al., 2007; Lam et al., 2014). Also, simultaneous manipulation of multiple genes by the gTME technique, mutagenesis, genome shuffling or adaptive evolution have been successfully applied (Alper et al., 2006; Demeke et al., 2013a; Voordeckers et al., 2015). However, these techniques alter many genes at once, which may have disadvantages for other important characterics of the strains. In addition, it is difficult to determine the relevant mutations contributing to the improved phenotype.

For the trait of high ethanol tolerance, we found that introduction of the superior alleles $ADE1_{Seg5}$, $KIN3_{Seg5}$; $MKT1_{VR1\text{-}5B}$ and $VPS70_{VR1\text{-}5B}$ individually or in combination could improve ER18. $ADE1_{Seg5}$ *and* $KIN3_{Seg5}$ were initially identified as causative alleles for maximal ethanol

accumulation. Ade1, N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase, is involved in *de novo* purine biosynthesis (Myasnikov et al., 1991) and Kin3, a non-essential serine/threonine kinase, is involved in DNA damage repair (Moura et al., 2010). Pais and co-workers (2013) described for the first time the connection of both alleles with ethanol production although no linkage was found with tolerance of cell proliferation to high ethanol levels. However, in our study, we have shown that allele replacement by the superior alleles $ADE1_{Seg5}$ and $KIN3_{Seg5}$ in ER18 could improve tolerance of cell proliferation to high levels of ethanol. Sequence analysis of the *ADE1* and *KIN3* alleles in ER18 revealed that that both alleles did not contain the unique mutations of the Seg5 strain (NC_001133:g.169227C>T (putative promoter of *ADE1*), NC_001133:g.171947C>T (putative promoter of *KIN3*) and NC_001133:g.170852C>T (ORF of *KIN3*)) as described in the study of Pais and co-workers (2013). Although the mutations in the ORF of *KIN3* are synonymous and mutations in the promoters of both alleles, it is plausible that they are affecting expression and thereby affecting ethanol tolerance and maximal ethanol accumulation in this way. Nevertheless, this hypothesis should be further investigated.

Allele replacement of the $MKT1_{VR1\text{-}5B}$- and $VPS70_{VR1\text{-}5B}$-*alleles* in the ER18 strain made the strain superior compared to the same strain with its own ER18 alleles for ethanol tolerance although $MKT1_{ER18}$ and $VPS70_{ER18}$ contain different mutations compared to those in the inferior alleles of the BY strain (Fig. 28). Mkt1 was initially reported as a posttranscriptional regulator of HO. Several previous studies identified *MKT1* in QTL mapping experiments in which the BY/S288c background was used as control strain, as causative allele for several stress-related complex phenotypes, like high temperature growth (Steinmetz et al., 2002), sporulation efficiency (Deutschbauer and Davis, 2005), induction of petite mutants (Dimitrov et al., 2009), drug resistance (Ehrenreich et al., 2010) and ethanol tolerance (Swinnen et al., 2012). Further research has shown that the D30G and K453R polymorphisms are responsible for deficiency of the Mkt1 protein in the BY strain (Lee et al., 2009). Also, Lewis and co-workers (2014) observed that *MKT1* affects a large number of transcripts responding to ethanol stress. Several studies showed that diploidization is triggered during long-term ethanol adaptation (Turanli-Yildiz et al., 2017; Voordeckers et al., 2015). In addition, Tannenbaum (2008) developed a mathematical model supporting the hypothesis that the diploid state in *S. cerevisiae* favours higher fitness and robustness to various growth and

environmental conditions. Turanli-Yildiz and co-workers (2017) showed that this diploidization triggered by long-term incubation in the presence of high ethanol lacked both specific polymorphisms, which means that this allele could also be superior for high ethanol tolerance. However, our results showed that the $MKT1_{ER18}$ allele is not defective but inferior to $MKT1_{VR1\text{-}5B}$ for ethanol tolerance. The $VPS70^{P199L}$ allele also appears to be important for high ethanol tolerance (Chapter II section 2.2; (Voordeckers et al., 2015). Our results show that $VPS70_{VR1\text{-}5B}$ is clearly superior compared to the $VPS70_{ER18}$ allele. Although both alleles contain the specific amino acid change P199L, there are other differences. Therefore, there may be other SNP(s) in the *VPS70* ORF contributing to high ethanol tolerance. It would be interesting to investigate to what extent the different SNPs contribute to this phenotype.

Introduction of $APJ1_{VR1\text{-}5B}$ or *apj1Δ* in the ER18 background did not cause an improvement of ethanol tolerance. The $APJ1_{VR1\text{-}5B}$-allele has a superior character over the BY-allele due to polymorphisms in the promoter and terminator regions (Chapter II section 2.3), which lead to lower expression of *APJ1* in the beginning of the fermentation (Swinnen et al., 2012). *APJ1* encodes a putative member of the Hsp40/DnaJ family of chaperone proteins, being involved in the regulation of the heat-shock protein Hsp70 and playing a role in degradation of sumolyated proteins (Cyr et al., 1994; Sahi et al., 2013). Hsp70 is important for nascent protein folding, complex remodelling and protein degradation (Meimaridou et al., 2009). Activity of Hsp70 is regulated by ATP binding and hydrolysis. Hsp40/DnaJ proteins have a conserved J domain that interacts with Hsp70 to accelerate ATP hydrolysis whereby Hsp70 adopts a high-affinity conformation towards its substrate (Greene et al., 1998; Langer et al., 1992). Apj1 is known to be present in extremely low abundance in the cell, which suggests that it may need to be localized properly in the cell in order to carry out its function (Ghaemmaghami et al., 2003). The $APJ1_{ER18}$-allele contained different SNPs in the promoter, terminator and ORF compared to the alleles from VR1-5B and S288c. However, no SNPs were located in the J domain, which is important for its activity. These results indicate that we cannot exclude a possible superior character of the ER18-allele for high ethanol tolerance and maximal ethanol accumulation.

For the trait of maximal ethanol accumulation capacity in VHG fermentation, we have found that the superior allele $VPS70_{VR1\text{-}5B}$ showed the largest contribution to the improvement of

the trait in the ER18 strain. This finding was rather unexpected because this allele was identified as a causative allele for ethanol tolerance of cell proliferation. However, when we look closely to the data of Pais and co-workers (2013), we could observe a possible minor QTL on chromosome X around the position of VPS70, both for maximal ethanol accumulation and tolerance of cell proliferation to high ethanol levels, but in the analysis, it was not statistically significant (appendix 4). On the contrary, $ADE1_{Seg5}$, $KIN3_{Seg5}$, which were identified as causative alleles for maximal ethanol accumulation (Pais et al., 2013), did not result in higher maximum ethanol levels in the ER18 strain. This was rather unexpected, however, maximal ethanol accumulation is a polygenic trait and interplay of different genes in the ER18 background could eliminate the superior character of the $ADE1_{Seg5}$ *and* $KIN3_{Seg5}$ alleles, although these alleles were found to be superior for ethanol tolerance of cell proliferation. Nevertheless, combination of $ADE1_{Seg5}$, $KIN3_{Seg5}$ and $VPS70_{VR1\text{-}5B}$ showed significant improvement of the maximal ethanol production levels.

In conclusion, our results show that introduction of the superior alleles $ADE1_{Seg5}$, $KIN3_{Seg5}$ *and* $VPS70_{VR1\text{-}5B}$ could improve ER18 for ethanol tolerance and maximum ethanol accumulation. These superior alleles may be the first choice to introduce in industrial yeast strains with low ethanol tolerance or low ethanol production capacity, especially in yeast strains with an ER background. Obviously, improvement of other industrial yeast strains with these superior alleles is not predictable, since the genetic background may play an important role.

Concluding remarks and perspectives

Worldwide, bioethanol is one of the commonly used biofuels. The yeast *Saccharomyces cerevisiae* is the most popular micro-organism used for bioethanol production due to its high ethanol tolerance, high ethanol productivity and ability to ferment a wide range of sugars. However, during the bioethanol production process, yeast encounters different stressors which inhibit ethanol production such as high ethanol concentrations, nutrient limitations and high temperature (Basso et al., 2008).

Causative genes involved in high ethanol tolerance of VR1-5B

In this study, we have further dissected the major QTL on chromosome X previously identified by polygenic analysis of the trait of high ethanol tolerance of cell proliferation in the bioethanol strain VR1 (Swinnen et al., 2012). We have determined the relevance and contribution of the different identified causative alleles for high ethanol tolerance in the inferior background S288c. We have to note that in the study of Swinnen and co-workers (2012), ethanol tolerance of cell proliferation was determined in rich YP medium with ethanol as sole carbon source. In this study, we have determined ethanol tolerance by scoring growth on solid rich medium with glucose as carbon source in the presence of ethanol. Scoring ethanol tolerance on YPD medium with different ethanol concentrations did not influence the results compared to the scoring of ethanol tolerance in the study of Swinnen and co-workers (2012). Moreover, malfunctioning alleles in one of the parental backgrounds compromising respiration would with our assay not be linked to the phenotype.

Swinnen and co-workers (2015) observed that different auxotrophic mutations reduce ethanol tolerance to different extents. The question remained why an auxotrophic mutation could affect ethanol tolerance. The plasma membrane is known to be one of the main targets of ethanol toxicity. The use of very high ethanol concentrations in our assays may have inhibited nutrient uptake across the plasma membrane. Therefore, an auxotrophic strain would be more compromised for growth in the presence of very high ethanol concentrations than a prototrophic strain. Our results have shown that auxotrophic mutations are not disadvantageous for growth in the presence of low to moderate ethanol concentrations but only at very high ethanol concentrations. In addition, we have shown that auxotrophic mutations are not disadvantageous for tolerance to other stress factors tested, i.e. acetic acid at pH 5, hydrogen peroxide and high temperature, except for osmotic stress caused by either

high glucose or high NaCl concentrations. Our results indicate that osmotic stress may have a similar inhibiting effect on the uptake of auxotrophic nutrients as high ethanol stress in rich medium. In general, our results suggest that stress factors inhibit the growth of auxotrophic strains depending on the growth conditions and that they do this by reducing the uptake of the auxotrophic nutrient to a level below what is required for a regular growth rate under the specific growth conditions used. Additionally, transport assays showed that the nutrient uptake rate was reduced with increasing ethanol concentrations. Our results showed that the uptake rate of uracil was less affected by high ethanol concentrations compared to that of leucine, but the absolute activity of uracil uptake was 500-fold lower than that of leucine. This may explain why uracil uptake in yeast is more sensitive to high ethanol concentrations than the uptake of amino acids. These results support the findings of Swinnen and co-workers (2015), that the *URA3* gene is much more strongly linked to very high ethanol tolerance than the amino acid biosynthesis genes *HIS3*, *LEU2*, *MET15* and *LYS2*. In conclusion, our results indicate that very high ethanol concentrations have a detrimental effect on plasma membrane transport, preventing sufficient nutrient uptake to fulfil auxotrophic requirements, and therefore auxotrophic mutations will lead to lower ethanol tolerance. Although the auxotrophic mutations clearly point to inadequate nutrient uptake as a possible cause of ethanol sensitivity, they are not directly useful for further improvement of ethanol tolerance in industrial yeast strains.

The dissection of the major QTL on chromosome X identified *VPS70* as the causative allele in this locus. Vps70 is a protein of unknown function involved in vacuolar protein sorting (Bonangelino et al., 2002). Previous research has implied that a properly functioning vacuolar system is required for ethanol tolerance (Meaden et al., 1999). Different genomic screens have associated genes involved in the vacuolar protein sorting machinery, vacuolar H^+-ATPase and vesicular transport to the vacuole with ethanol tolerance (Auesukaree et al., 2009; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006). The present work has revealed the first link of *VPS70* to high ethanol tolerance. Recently, another group has identified the same mutation, $VPS70^{C595A}$, in an ethanol-tolerant strain obtained by evolutionary adaptation. It provided the largest fitness increase under high ethanol conditions (Voordeckers et al., 2015). Both studies indicate that *VPS70* indeed plays a role in ethanol tolerance. Although the vacuolar system is important for ethanol tolerance, we

cannot ignore the fact that Vps70 is located in the endoplasmic reticulum (Yofe et al., 2016). The endoplasmic reticulum is a cell organelle where secreted proteins are folded and membrane lipids are synthesized. Ethanol stress is known to cause unfolding of proteins and disturbs membrane lipid homeostasis. Recently, Brown and co-workers (2013) suggested that the unfolded protein response (UPR) is involved in ethanol tolerance because the UPR is induced during the bioethanol fermentation process in highly ethanol tolerant strains as opposed to the laboratory strain S288c. Likewise, Miyagawa and co-workers (2014) propose that ethanol stress compromises ER protein folding and induces the UPR which contributes to high ethanol tolerance. Although the function of Vps70 is still unknown, the *VPS70* allele derived from the VR1-5B strain seems to be an interesting candidate to improve other yeast strains for high ethanol tolerance.

Swinnen and co-workers (2012) identified *APJ1* as a causative gene for high ethanol tolerance. They found that the presence of the VR1-5B allele from *APJ1* gene resulted in higher ethanol tolerance compared to the BY4741 allele in the heterozygous background BY/VR1-5B. In addition, deletion of *APJ1* in both of the parental backgrounds resulted in better growth on high ethanol concentrations than the wild type strains. These results all together suggested that the *APJ1* gene product acts negatively on ethanol tolerance, at least under our test conditions. And the fact that there are no nonsynonymous mutations in the ORF of *APJ1* between both parents suggested that a difference in expression level of the parental alleles may account for the difference in ethanol tolerance. Real-time PCR analysis showed that *APJ1* expression in the beginning of the fermentation was about 2.5-fold higher in the BY4741 strain than in the VR1-5B strain. However, it cannot be ignored that the conditions of expression analysis and the growth experiments on solid nutrient medium are different, therefore, it is not possible to make a definite conclusion.

From our results, we suggest that the superior character of the VR1-5B allele of *APJ1* is due to its lower expression compared to the BY4741 derived allele, and that the *APJ1* gene product has a negative effect on ethanol tolerance. Improvement of industrial yeast strains for ethanol tolerance of cell proliferation on solid nutrient medium by deletion of *APJ1* was not always possible, which means that changing one genetic determinant does not necessarily lead to improvement of the phenotype of interest.

As a result of the first part of the thesis and previous research (Swinnen et al., 2012), we have managed to analyze all the major QTLs found for high ethanol tolerance in the former Brazilian bioethanol production strain VR1 versus the S288c control strain and identified the causative genes *URA3*, $VPS70_{VR1\text{-}5B}$, $MKT1_{VR1\text{-}5B}$, $SWS2_{BY4741}$ and $APJ1_{VR1\text{-}5B}$. As a proof of principle, we have introduced all causative genes in the inferior background strain S288c. *MKT1* conferred the highest contribution to ethanol tolerance followed by *VPS70* and *APJ1* deletion. The triple combination of the superior alleles in S288c resulted even in a higher ethanol tolerance than that of the superior parent VR1-5B. This indicates that the inferior strain can contain additional interesting mutations that to some extent can contribute to the phenotype of interest.

Causative genes involved in high ethanol accumulation capacity of Seg5

In the first part of this thesis, we determined ethanol tolerance in terms of the inhibition of growth on solid nutrient medium by increasing ethanol levels. This is less relevant for industrial fermentations in which maximal ethanol accumulation, high fermentation rate and maintenance of cell viability is most important. In the second part of the thesis the focus was therefore on identifying new genes involved in high ethanol accumulation capacity in VHG fermentations. Up till now, many research groups have done functional genomic screens for ethanol tolerance using single gene knockout collections (Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). However, only one research group has applied this functional genomic screen to study the completion of fermentation in high sugar medium, in which multiple stresses are experienced such as high osmolarity and high ethanol concentrations (Walker et al., 2014).

We have found new causative alleles in the QTLs on Chr. X and Chr. XV, identified by polygenic analysis of maximal ethanol accumulation capacity in the sake strain CBS1585 (Pais et al., 2013). Detailed analysis of QTL 9 on Chr. X and QTL 17 on Chr. XV revealed two genes, *PTK2* and *SHE4* respectively, linked to maximal ethanol accumulation capacity, of which the latter was also linked to high ethanol tolerance of cell proliferation on solid nutrient medium. *PTK2* encodes a serine/threonine protein kinase, which is involved in the regulation of ion transport across the plasma membrane. *SHE4* is involved in actin cytoskeleton polarization. Both genes

have already been associated previously with ethanol tolerance in *S. cerevisiae* (Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). Besides Walker and colleagues (2014) also identified *PTK2* as one of the 93 fermentation essential genes (FEG). *PTK2* is an interesting causative gene because it is essential for glucose-dependent activation of Pma1. The plasma membrane H^+-ATPase Pma1 ensures the strength of the proton electrochemical gradient and must be precisely regulated so that cells can transport nutrients, regulate osmotic balance, ion homeostasis and stress tolerance (Pereira et al., 2015). Several studies have demonstrated that overexpression of *PMA1* enhances ethanol tolerance and yeast performance in VHG fermentations (Lam et al., 2014; Lee et al., 2017). Sequence analysis of the Seg5 and BY4742-derived alleles from *PTK2* showed two amino acid changes in the catalytic domain (central part of the enzyme extending from amino acid 261 to 562; (Eraso et al., 2006)), other amino acid changes were mainly at the N-terminus of the protein. These mutations could be beneficial for protein stability and activity under conditions of ethanol stress, but this hypothesis needs further investigation.

The *SHE4* allele from the low ethanol tolerant parent strain was beneficial for maximal ethanol accumulation capacity and ethanol tolerance of cell proliferation. Many studies have shown that defects in major structures of the cell, like the cell wall or actin cytoskeleton, result in increased sensitivity to multiple stresses (Auesukaree et al., 2009; Chowdhury et al., 1992; Kubota et al., 2004). Surprisingly, the causative allele of *SHE4* was linked to the inferior parent. This QTL or the causative gene were not found to be linked in the previous study on high ethanol tolerance of cell proliferation in a former bioethanol production strain although this study was performed with practically the same inferior strain. This could have two possible reasons. On the one hand, there were no important polymorphisms between the superior VR1-5B strain and the inferior BY4741 strain in this gene, resulting in a 50% segregation and therefore no linkage to the phenotype in the QTL mapping. On the other hand, the Seg5-allele of *SHE4* has a negative effect on high ethanol accumulation capacity and ethanol tolerance of cell proliferation. Further research is needed to distinguish between the two possibilities.

Global transcriptomic analysis can provide an overall view of genes possibly involved in ethanol tolerance or affected by the response to ethanol stress. We have performed pooled-

segregant gene expression analysis at a certain fermentation time-point in order to find target genes that might be candidate genes for strain improvement by maximizing ethanol accumulation capacity. Five out of 35 differentially expressed target genes on a multi-copy vector pVV214 increased ethanol accumulation capacity of the reference strain V1116. However, integration of the overexpression cassettes in the genome of V1116 gave no improvement. Chromosomal integration of the overexpression cassette allows for more precise control of the gene copy number via constitutive or inducible promotors (Da Silva and Srikrishnan, 2012). Even if integration may not be perfect for precise overexpression of the candidate gene, it is necessary to obtain a stable yeast strain. We believe that the combination of QTL mapping and transcriptome analysis holds great potential for understanding the molecular mechanisms underlying complex traits. However, our experimental set-up had some drawbacks. First, we did not obtain transcriptome data of the inferior and superior strain and selected pool of segregants in non-stressed conditions (without ethanol, and with a low sugar concentration). Those data could provide information on differentially expressed genes that are not linked to the phenotype but rather play a role in overall fitness of the strain. Hence, it is possible that our top list of overexpressed genes contains genes that are differentially expressed between the two parent strains, not because of their difference in ethanol tolerance but because of a difference in general fitness under the conditions that we have used to measure the ethanol tolerance. Second, complex traits such as maximal ethanol accumulation capacity are conferred by multiple genes acting together. Therefore, introduction of all identified target genes together in a single yeast strain could increase the chances of improving the phenotype of interest compared to introduction of single target gene changes.

Improvement of ER18 for ethanol tolerance and maximal ethanol accumulation capacity

In the present study, we have used ER18 as the base strain for providing a proof-of-principle for improvement of ethanol tolerance of cell proliferation and maximal ethanol accumulation capacity in high gravity fermentations. Combined introduction of $ADE1_{Seg5}$, $KIN3_{Seg5}$ and $VPS70_{VR1\text{-}5B}$ resulted in the largest improvement of ethanol tolerance of cell proliferation and maximal ethanol accumulation capacity in ER18. Those three genes are involved in purine synthesis, DNA repair and vacuolar protein sorting, which are all three functional categories linked to different stresses regularly encountered during fermentation. *ADE* genes were

previously linked to high sugar tolerance (Ando et al., 2006), ethanol is known to cause DNA damage (Ibeas and Jimenez, 1997; Ristow et al., 1995) and ethanol affects vesicular transport and vacuole morphology (Meaden et al., 1999). Still, it would be interesting to investigate the contribution of the newly identified causative alleles $PTK2_{Seg5}$ and $SHE4_{BY}$ for high ethanol tolerance and maximal ethanol production capacity in this background. ER18, however, is a segregant of Ethanol Red and because it is a relatively weak haploid strain, this represents already a limitation for the possibility of transferring the results obtained for these traits to different industrial yeast strains and successfully obtaining the desired improvement of the phenotype(s).

Future perspectives

Pooled-segregant whole-genome sequence analysis has proven to be a successful approach for studying important industrial traits such as high ethanol tolerance or maximal ethanol accumulation capacity. However, with this technology we are only studying the genetic differences of the trait under investigation between a certain superior strain and inferior in order to identify causatives genes. In this work, we succeeded in identifying new causative genes for both traits, *VPS70* (high ethanol tolerance), *PTK2* and *SHE4* (maximal ethanol accumulation). Those superior alleles can now be used to improve both traits in other industrial yeast strains. However, caution must be taken before replacing these genes directly in the industrial yeast strains. These genes have been identified as important genetic differences between an industrial strain and a commonly used laboratory strain. Thereby, other industrial yeast strains could also contain a superior allele of the causative genes. What is more, replacing an inferior allele by a superior allele does not necessarily lead to an improved phenotype because of the polygenic nature of the trait, which thus depends on many interacting genes and therefore depends on the strain background. Therefore, it would be interesting to investigate if replacement of all causative ethanol tolerant alleles in a moderately ethanol tolerant strain would further improve this trait instead of selecting an inferior strain such as ER18.

Furthermore, it would be interesting to extend this QTL analysis to other yeast strains with high ethanol tolerance or maximal ethanol accumulation capacity in order to investigate if the same or different genes are involved. In this way, we could determine if the identified

causative genes are universal determinants for the trait under investigation and gain more insight in the common genetic basis of ethanol tolerance or maximal ethanol accumulation capacity in different strains. It is important to score the traits under the same experimental conditions. Previous research by functional genomic screens of ethanol tolerance has shown limited overlap in the results likely due to differences in strain backgrounds and experimental conditions (Fujita et al., 2006; Kubota et al., 2004; Teixeira et al., 2009; van Voorst et al., 2006; Yoshikawa et al., 2009). Moreover, if research initiated by Swinnen and co-workers (2012) and Pais and co-workers (2013) on ethanol tolerance and maximal ethanol accumulation capacity would have been performed under the same selection conditions, we could have studied whether the same QTLs were linked to these traits (Appendix 4 and 5).

Conclusions

Altogether, this work has provided new insights into the genetic basis of high ethanol tolerance of cell proliferation and maximal ethanol accumulation capacity of industrial yeast strains. All causative quantitative trait genes (*URA3*, *VPS70*, *MKT1* and *APJ1*) of the major QTLs for high ethanol tolerance in VR1-5B have now been identified. For maximal ethanol accumulation capacity two new causative genes (*PTK2* and *SHE4*) were identified. Overall, all these identified causative genes are involved in cellular processes that counteract ethanol toxicity (Fig. 38). Introduction of a combination of specific causative genes in ER18 could clearly improve this strain for both phenotypes. However, it still remains to be investigated to what extent these causative genes could be universally used to improve other industrial yeast strains for bio-ethanol production.

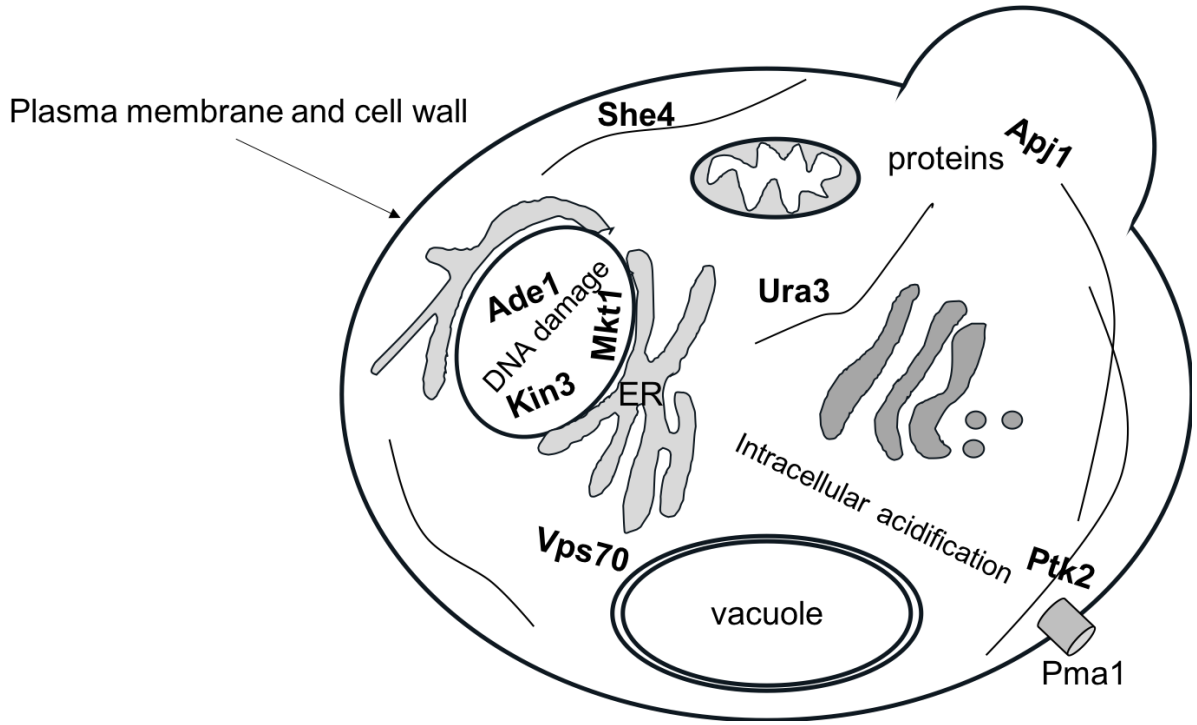


Figure 38: Summary of identified causative genes by pooled-segregant whole-genome sequence analysis of the traits high ethanol tolerance and maximal ethanol accumulation capacity in Saccharomyces cerevisiae.

Materials and methods

1.1 Yeast strains and growth conditions

All yeast strains were grown in yeast extract-peptone dextrose (YPD) broth containing 1% (w/v) yeast extract, 2% (w/v) Bacto peptone and 2% (w/v) glucose at 30°C and 200 rpm. For solid medium (YPD plates), 1.5% agar was added. Selection of positive transformants was done on YPD plates supplemented with antibiotic, being 200 µg/mL geneticin, 100 µg/mL nourseothricin, 300 µg/mL hygromycin B and/or 15 µg/mL phleomycin. Ethanol tolerance assays were performed on YP and YPD plates containing ethanol at the indicated concentrations (vol/vol). Other stress tolerance assays were performed on YPD plates containing 0.3% or 0.4% (vol/vol) acetic acid at pH 5; 4.0 mM or 4.5 mM H_2O_2; 1.50 M or 1.75 M NaCl; and 25% or 30% glucose. Stress tolerance assays with limiting amounts of either uracil or leucine were performed on minimal medium containing a complete synthetic dropout amino acid-nucleotide mixture (CSM-AA; amounts according to the manufacturer's instructions) containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 1.5% agar, and different amounts of uracil (0.005 to 0.400 mM) or leucine (0.05 to 2.00 mM), respectively. The pH of the medium was adjusted to 6.5 with 4 M KOH. After autoclaving, different concentrations of ethanol or NaCl were added to the medium as indicated. Transport assays were performed in minimal medium with 2% glucose or yeast nitrogen base (YNB) medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose. The pH of YNB medium was adjusted to 4.8 with HCl. All concentrations mentioned above are in weight per volume unless indicated differently. Yeast strains used in this study are listed in table 8 in section 1.13.

1.2 General molecular biology methods

Yeast genomic DNA was extracted with TE buffer (pH 7.8) and PCI (25:24:1) according to Hoffman and Winston (1987) and further purified with ethanol precipitation, if required. Polymerase chain reaction (PCR) was performed with high-fidelity polymerase Q5 (New England Biolabs) for cloning and sequencing purposes and with Taq (New England Biolabs) for diagnostic purposes. Sanger sequencing (Sanger and Coulson, 1975) was performed by the Genetic Service Facility (Antwerp, Belgium) of the VIB. The sequences were analysed with CLC DNA workbench (CLC Bio) software (Qiagen). Yeast cells were transformed with the LiAc/PEG method (Gietz et al., 1995). Cloning was performed by standard techniques.

Dephosphorylation was performed with rAPid Alkaline Phosphatase (Roche) and ligation with T4 DNA ligase (Roche). *E. coli* transformation was performed with the $CaCl_2$ method (Sambrook et al. 1989) and plasmid DNA was isolated from *E. coli* by Miniprep (Sigma). The plasmids and primers used in this study are listed in table 9 and 10 in section 1.13.

1.3 Plasmid construction by Gateway™ cloning technology

Gateway™ recombinational cloning technology was used to clone candidate genes into the high copy expression vector pVV214-hph. Plasmids used are listed in table 9 in section 1.13. Candidate genes were amplified by PCR from the superior parent Seg5 flanked with attB1 and attB2 sites. Primers used are listed in table 10 in section 1.13. The attB-flanked PCR product was cloned into the destination vector pDONR201 by the BP Clonase™ II enzyme to generate an entry clone bearing the candidate gene. Thereafter, the candidate gene was cloned into the destination vector pVV214-hph by the LR Clonase™ II enzyme, resulting is the expression vector with the ORF of interest.

1.4 Genotyping of SNP by allele-specific PCR

For each SNP, at a given chromosomal position, two primers, either in the forward or reverse direction, were constructed with one mismatch at their 3'-terminal ends. In particular, one primer contained the parent-1 nucleotide, while the other primer contained the parent-2 nucleotide. Both primers were always applied in separate PCR reactions with a common primer. The annealing temperature was optimized by gradient PCR and using genomic DNA of both parents to allow only hybridization with primers containing the exact complement. Reactions were performed in 20 µL mixtures containing 10 ng template DNA, 10 pmol of forward and reverse primers, and appropriate amounts of dNTPs, Taq polymerase, and Standard Taq buffer, according to the manufacturer's guidelines (New England Biolabs). The annealing temperature at which the best distinguishing reaction was obtained with the two parents was used for scoring the SNPs in the individual segregants for highly ethanol-tolerant or maximal ethanol production segregants. The parental strains were used as positive and negative control, depending of the pair of primer used. Primers used are listed in table 10 in section 1.13.

1.5 (Bulk) Reciprocal Hemizygosity Analysis

For (bulk)RHA analysis (Steinmetz *et al.* 2002) the exact ORFs (RHA) or large blocks (bulk RHA) were deleted with the maker NatMX or KanMX in the VR1-5B/BY4741 or BY4742/Seg5 diploid strains. The transformants were selected on YPD plates supplemented with nourseothricin or geneticin. The correct integration of the deletion cassette was verified by PCR and the remaining, i.e. non-deleted, allele/block was determined by allele-specific PCR. Primers used are listed in table 10 in section 1.13. (Bulk)RHA analysis was performed with 2 or 3 independent transformants for all hybrids tested.

1.6 Stress tolerance spot assays

Cells taken from solid YPD medium were transferred into 3 ml YPD broth or minimal medium in a glass tube and cultivated for 3 days in an orbital shaker at 30°C and 200 rpm. The cultures were then diluted in water to OD_{600} 0.5 and further serially diluted to obtain a 2-fold (10^0 until 8×10^{-3}) or 10-fold (10^0 until 10^{-3}) dilution range. From each dilution, 5 µL was spotted onto solid YPD and YP, or minimal medium containing different stress factors, as indicated. Growth was scored after 1 day for control YPD plates and after 2 to 11 days, as indicated, for plates with different concentrations of ethanol or other stress factors. All plates were incubated at 30°C, except for the heat stress tolerance assay, in which case the plates were incubated at 39°C or 40°C.

1.7 Real-time PCR

For measurement of *APJ1* expression, samples were taken from early exponential-phase grown cells of BY4741 and VR1-5B. Pellets were frozen in liquid nitrogen and stored at -80°C. RNA extraction was performed using the phenol-chloroform method and treated with RNAse free DNAse (Roche). cDNA was prepared following the instructions of the GoScript Reverse Transcription System kit (Promega). Relative quantification of *APJ1* and *18S* was performed using a StepOnePlus Real-time PCR system (Applied Biosystems), primers: Fw *APJ1* (TGATGGGCACGGTGGTCTA), Rv *APJ1* (TTGAATACCTTGCCCTTTGCA), Fw *18S* (CACTTCTTAGAGGGACTATCGGTTTC) and Rv *18S* (CAGAACGTCTAAGGGCATCACA).

1.8 Very high gravity Fermentations (small scale)

Cells were pre-grown in 3 mL YPD at 30°C and 200 rpm for 24h. Cells of the pre-culture were inoculated in 100 mL of YP + 10% (w/v) glucose with an initial OD_{600} of 1. For the strains carrying the plasmid pVV214-geneX, hygromycin B (300 µg/mL) was added to the YPD medium to maintain the plasmid in the strains. The cells were grown at 30°C and 200 rpm for 2 days until stationary phase. Cells were harvested and used to inoculate 100 ml YP ±33% (w/v) glucose with an initial OD_{600} of 4, unless otherwise stated. The fermentation cultures were carried out semi-static and incubated at 25, 30 or 32°C depending on the experiment, agitation was done with a magnetic rod at 200 rpm for 4h. The fermentation profile was followed from weight loss due to CO_2 release at different time intervals during the fermentation period. Samples were taken at the end of the fermentation for HPLC analysis.

1.9 Analysis of metabolites by HPLC

Metabolites in samples from fermentation experiments were analysed by HPLC (Waters Isocratic Breeze system, Milford, MA, USA) using ion-exchange column WAT010290 at 75°C and a refractive index detection system (Waters 2414 RI detector). 5 mM H_2SO_4 was used as eluent at a flow rate of 1 mL/min. At the end of the fermentation, 15 mL-samples were taken and centrifuged at 3000 rpm for 10 min. Then, 800 µL of the samples was transferred to a HPLC vial of which 10 µL was automatically injected and processed for 20 min. The metabolites quantified by HPLC were glucose, glycerol, acetic acid and ethanol.

1.10 Transport of amino acids and bases

Cells of the S288c, S288c *ura3-52*, and S288c *leu2Δ* strains were cultivated in 5 mL of minimal medium in an orbital shaker at 30°C, 200 rpm and overnight. Each pre-culture was then used to inoculate 200 mL of YNB medium containing 2 % glucose (pH 4.8). The cells were grown to an OD_{600} of 1 to 2, after which they were harvested by centrifugation (3,000 rpm for 5 min), washed twice with morpholineethanesulfonic acid (MES)-KOH (25 mM; pH 6), and resuspended in fresh YNB medium to a final cell concentration of 160 mg ml^{-1}. Of this cell suspension, 50 µL was transferred to a new centrifuge tube and incubated for 10 min at 30°C. In order to start the transport measurements, a volume of 40 µL a specific ethanol concentration was first added to the cell suspension to reach a final ethanol concentration of 5; 10; 14 or 18% (v/v). After 5 min of incubation, 10 µL of either ^{3}H-labeled uracil to a final

concentration of 0.05 mM (PerkinElmer) or ^{14}C-labeled L-leucine to a final concentration of 2.5 mM (PerkinElmer) was added to the suspension. After 2 min of incubation, 5 ml of ice-cold water was added to stop the uptake of the radiolabelled nutrients. The cells were collected by using a glass microfiber filter (Whatman GF/C, with a retention particle size of 1.2 µm), saturated with unlabelled nutrient solution, and immediately washed twice with 5 ml of ice-cold water. For both transport assays (uracil and leucine), three samples with radiolabelled nutrient and two blanks (addition of 5 ml of ice-cold water followed by the addition of radiolabelled nutrient) were analysed. The radioactivity on the filter was determined with a liquid scintillation counter (LS6500; Beckman Coulter). A 500 µL aliquot of the cell suspension was taken to determine protein content by using the Bradford method (Bradford, 1976). Transport activity was expressed as nanomole substrate transported per minute and milligram of protein and as a percentage of the transport rate in the absence of ethanol.

1.11 Allele replacement

1.11.1 Tools for PhiC31-mediated gene replacement in yeast

Allele replacement in S288c was achieved by a two-step genetic marker-free approach mediated by the Phi-C31 integrase system. In the first step, the ORF was deleted with a deletion cassette amplified by PCR, containing the selectable marker KanMX flanked by upstream the attB (ccgcggtgcgggtgccagggcgtgcccTTGggctccccgggcgcgtactccac) and A1 (GTGGTCGGCTGGAGATCGG) sites and downstream the attP (gtagtgccccaactggggtaacctTTGagttctctcagttgggggcgtag) and A2 (AGCCGTTATGGCGGGCATC) sites. The priming sites (A1 and A2) were included to amplify the linear DNA allowing the use of primers with 50 bp tail matching with the target site for homologous recombination and resulting in the integration of the cassette in the desirable place. Positive transformants were selected on YPD geneticin plates. The presence of the deletion cassette was verified by PCR. In the second step, the superior allele fused with NatMX antibiotic resistance marker flanked with attB and attP sites was introduced at the original locus in the genome. Positive transformants were selected on YPD nourseothricin plates. In the final step, the antibiotic marker was removed using the Phi-C31 integrase and leaving a small scar (attL (ccgcggtgcgggtgccagggcgtgcccTTGagttctctcagttgggggcgtag) site) in the region concerned in

the genome. The presence of the superior allele was verified with PCR and allele specific PCR. Plasmids and primers used are listed in table 9 and 10 in section 1.13.

1.11.2 Tools for Cas9-mediated gene replacement in yeast

Gene replacement in ER18 by CRISPR/Cas9 technology was performed using the pTEF-Cas9-BLE plasmid (single copy, Cas9 behind the TEF1 promoter, Ble marker), which was derived from the p414-TEF1p-Cas9-CYC1t plasmid (Dicarlo *et al*., 2013) by replacing *URA* marker by Ble cassette for its selection. The gRNA vector, named pgRNA-G2-HPH, contained the hygromycin marker (HPH) and 20 nt spacer (G2, GGATGAGAATCTGACAAAGGTGG) that defines a specific target sequence. The antibiotic markers used for knocking out the endogenous alleles were flanked by the G2-gRNA recognition site and the priming sites A1 and A2. Plasmids used are listed in table 9 in section 1.13. Donor DNA was PCR amplified from the superior parent VR1-5B or Seg5. First, the original allele was replaced by an antibiotic marker flanked with two G2-gRNA recognition sites. Second, cells were transformed with the pTEF-Cas9-BLE plasmid by heat-shock (Gietz et al., 1995). Cas9 transformants were transformed with 500 ng of the G2-gRNA plasmid and 1000 ng of donor DNA (PCR product). Transformants were selected on solid YPD media supplemented with hygromycin (300 µg/mL). Allele replacement was verified by allele-specific PCR.

1.12 Calculations, reproducibility and statistical analysis

All fermentations and allele replacement were carried out in triplicate, unless stated otherwise. The mean of the fermentation data is presented in bar charts. The error bars always represent one standard deviation. The results were analysed by unpaired Student's t-test or by one-way ANOVA with Dunnett's correction.

1.13 Yeast strains, plasmids and primers

Table 8: S. cerevisiae strains used in this study.

Strain	Genotype/description	Reference
BY4741	*Mat**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachman et al., 1998
BY4742	*Mat his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachman et al., 1998
S288c (Mata)	*Mat**a*** prototroph	Brachman et al., 1998
S288c (Mat)	*Mat* prototroph	Brachman et al., 1998
S288c *ura3-52Δ*	*Mat**a** ura3-52*	Grundmann et al., 2001
S288c *leu2Δ*	*Mat**a** leu2Δ::attR*	this study
BY4741/VR1-5B	Hybrid diploid, BY4741/VR1-5B	this study
BY4742/Seg5	Hybrid diploid, BY4742/Seg5	this study
VR1-5B	Haploid (*Mat*) segregant from VR1, with similar high ethanol tolerance	Swinnen et al., 2012
Seg5	Haploid (Mata) segregant from CBS1585, with similar maximal ethanol accumulation	Pais et al., 2013
BY *yir120WΔ*/VR	Hybrid diploid BY4741/VR1-5B *yir120W*^{BY4741}*::KanMX / YIR120W*$^{VR1-5B}$	this study
BY /VR *yir120WΔ*	Hybrid diploid BY4741/VR1-5B *YIR120W*^{BY4741} */ yir120W*$^{VR1-5B}$*::KanMX*	this study
BY *yjr124CΔ*/VR	Hybrid diploid BY4741/VR1-5B *yjr124C*^{BY4741}*::KanMX / YJR124C*$^{VR1-5B}$	this study
BY /VR *yjr124CΔ*	Hybrid diploid BY4741/VR1-5B *YJR124C*^{BY4741} */ yjr124C*$^{VR1-5B}$*::KanMX*	this study
BY *jhd2Δ*/VR	Hybrid diploid BY4741/VR1-5B *jhd2*^{BY4741}*::KanMX / JHD2*$^{VR1-5B}$	this study
BY /VR *jhd2Δ*	Hybrid diploid BY4741/VR1-5B *JHD2*^{BY4741} */ jhd2*$^{VR1-5B}$*::KanMX*	this study
BY *rsf2Δ*/VR	Hybrid diploid BY4741/VR1-5B *rsf2*^{BY4741}*::KanMX / RSF2*$^{VR1-5B}$	this study
BY /VR *rsf2Δ*	Hybrid diploid BY4741/VR1-5B *RSF2*^{BY4741} */ rsf2*$^{VR1-5B}$*::KanMX*	this study
BY *vps70Δ*/VR	Hybrid diploid BY4741/VR1-5B *vps70*^{BY4741}*::KanMX / VPS70*$^{VR1-5B}$	this study
BY /VR *vps70Δ*	Hybrid diploid BY4741/VR1-5B *VPS70*^{BY4741} */ vps70*$^{VR1-5B}$*::KanMX*	this study
VR1-5B *vps70Δ*	*vps70::KanMX*	this study
BY4741 *vps70Δ*	*vps70::KanMX*	this study
VR1 (A)	Segregant A of one complete tetrad of VR1	F. Dumortier
VR1 (B)	Segregant B of one complete tetrad of VR1 = VR1-5B	F. Dumortier
VR1 (C)	Segregant C of one complete tetrad of VR1	F. Dumortier
VR1 (D)	Segregant D of one complete tetrad of VR1	F. Dumortier
ER-1A	Segregant 1A of one complete tetrad of ER	this study
ER-1C	Segregant 1C of one complete tetrad of ER	this study
PE2-1A	Segregant 1A of one complete tetrad of PE2	this study
PE2-1B	Segregant 1B of one complete tetrad of PE2	this study
ES2-1A	Segregant 1A of one complete tetrad of ES2	this study
ES2-1B	Segregant 1B of one complete tetrad of ES2	this study
CAT1-1B	Segregant 1B of one complete tetrad of CAT1	this study
CAT1-1C	Segregant 1C of one complete tetrad of CAT1	this study
ER	Ethanol Red, bioethanol production	Lesaffre, France
ER *apj1Δ*	Single knock-out of *APJ1* in ER, *apj1::loxP*	this study
ER *apj1ΔΔ*	Double knock-out of *APJ1* in ER, *apj1::loxP*	this study

PE2	Bioethanol production	Fermentec, Brazil
PE2 *apj1Δ*	Single knock-out of *APJ1* in PE2, *apj1::loxP*	this study
PE2 *apj1ΔΔ*	Double knock-out of *APJ1* in PE2, *apj1::loxP*	this study
CAT1	Bioethanol production	Fermentec, Brazil
CAT1 *apj1Δ*	Single knock-out of *APJ1* in CAT1, *apj1::loxP*	this study
CAT1 *apj1ΔΔ*	Double knock-out of *APJ1* in CAT1, *apj1::loxP*	this study
ES2	Fali ES2, wine strain	AB Mauri, Australia
ES2 *apj1Δ*	Single knock-out of *APJ1* in ES2, *apj1::loxP*	this study
ES2 *apj1ΔΔ*	Double knock-out of *APJ1* in ES2, *apj1::loxP*	this study
S288c *MKT1*	S288c $mkt1^{S288C}$*::*$MKT1^{VR1\text{-}5B}$	this study
S288c VPS70	S288c $vps70^{S288C}$*::*$VPS70^{VR1\text{-}5B}$	this study
S288c *apj1Δ*	S288c $apj1^{S288C}$*::KanMX*	this study
S288c *MKT1 VPS70*	S288c $mkt1^{S288C}$*::*$MKT1^{VR1\text{-}5B}$ $vps70^{S288C}$*::*$VPS70^{VR1\text{-}5B}$	this study
S288c *MKT1 apj1Δ*	S288c $apj1^{S288C}$*::KanMX* $mkt1^{S288C}$*::*$MKT1^{VR1\text{-}5B}$	this study
S288c *VPS70 apj1Δ*	S288c $apj1^{S288C}$*::KanMX* $vps70^{S288C}$*::*$VPS70^{VR1\text{-}5B}$	this study
S288c *MKT1 VPS70 apj1Δ*	S288c $apj1^{S288C}$*::KanMX* $mkt1^{S288C}$*::*$MKT1^{VR1\text{-}5B}$ $vps70^{S288C}$*::*$VPS70^{VR1\text{-}5B}$	this study
BY *block1Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block1(chr.X)*^{BY4742}*::KanMX /* *Block1(chr.X)*^{Seg5}	this study
BY/Seg5 *block1Δ*	Hybrid diploid BY4742/Seg5 *Block1(chr.X)*^{BY4742} */* *block1(chr.X)*^{Seg5}*::KanMX*	this study
BY *block2Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block2(chr.X)*^{BY4742}*::KanMX /* *Block2(chr.X)*^{Seg5}	this study
BY/Seg5 *block2Δ*	Hybrid diploid BY4742/Seg5 *Block2(chr.X)*^{BY4742} */* *block2(chr.X)*^{Seg5}*::KanMX*	this study
BY *block3Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3(chr.X)*^{BY4742}*::KanMX /* *Block3(chr.X)*^{Seg5}	this study
BY/Seg5 *block3Δ*	Hybrid diploid BY4742/Seg5 *Block3(chr.X)*^{BY4742} */* *block3(chr.X)*^{Seg5}*::KanMX*	this study
BY *block4Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block4(chr.X)*^{BY4742}*::KanMX /* *Block4(chr.X)*^{Seg5}	this study
BY/Seg5 *block4Δ*	Hybrid diploid BY4742/Seg5 *Block4(chr.X)*^{BY4742} */* *block4(chr.X)*^{Seg5}*::KanMX*	this study
BY *block3.1Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3.1(chr.X)*^{BY4742}*::KanMX /* *Block3.1(chr.X)*^{Seg5}	this study
BY/Seg5 *block3.1Δ*	Hybrid diploid BY4742/Seg5 *Block3.1(chr.X)*^{BY4742} */* *block3.1(chr.X)*^{Seg5}*::KanMX*	this study
BY *block3.2Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3.2(chr.X)*^{BY4742}*::KanMX /* *Block3.2(chr.X)*^{Seg5}	this study
BY/Seg5 *block3.2Δ*	Hybrid diploid BY4742/Seg5 *Block3.2(chr.X)*^{BY4742} */* *block3.2(chr.X)*^{Seg5}*::KanMX*	this study
BY *block3.3Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3.3(chr.X)*^{BY4742}*::KanMX /* *Block3.3(chr.X)*^{Seg5}	this study
BY/Seg5 *block3.3Δ*	Hybrid diploid BY4742/Seg5 *Block3.3(chr.X)*^{BY4742} */* *block3.3(chr.X)*^{Seg5}*::KanMX*	this study
BY *block3.4Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3.4(chr.X)*^{BY4742}*::KanMX /* *Block3.4(chr.X)*^{Seg5}	this study
BY/Seg5 *block3.4Δ*	Hybrid diploid BY4742/Seg5 *Block3.4(chr.X)*^{BY4742} */* *block3.4(chr.X)*^{Seg5}*::KanMX*	this study
BY *block1Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block1(chr.XV)*^{BY4742}*::KanMX /* *Block1(chr.XV)*^{Seg5}	this study
BY/Seg5 *block1Δ*	Hybrid diploid BY4742/Seg5 *Block1(chr.XV)*^{BY4742} */* *block1(chr.XV)*^{Seg5}*::KanMX*	this study
BY *block2Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block2(chr.XV)*^{BY4742}*::KanMX /* *Block2(chr.XV)*^{Seg5}	this study

BY/Seg5 *block2Δ*	Hybrid diploid BY4742/Seg5 *Block2(chr.XV)^{BY4742} / block2(chr.XV)^{Seg5}::KanMX*	this study
BY *block3Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block3(chr.XV)^{BY4742}::KanMX / Block3(chr.XV)^{Seg5}*	this study
BY/Seg5 *block3Δ*	Hybrid diploid BY4742/Seg5 *Block3(chr.XV)^{BY4742} / block3(chr.XV)^{Seg5}::KanMX*	this study
BY *block4Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block4(chr.XV)^{BY4742}::KanMX / Block4(chr.XV)^{Seg5}*	this study
BY/Seg5 *block4Δ*	Hybrid diploid BY4742/Seg5 *Block4(chr.XV)^{BY4742} / block4(chr.XV)^{Seg5}::KanMX*	this study
BY *block5Δ*/Seg5	Hybrid diploid BY4742/Seg5 *block5(chr.XV)^{BY4742}::KanMX / Block5(chr.XV)^{Seg5}*	this study
BY/Seg5 *block5Δ*	Hybrid diploid BY4742/Seg5 *Block5(chr.XV)^{BY4742} / block5(chr.XV)^{Seg5}::KanMX*	this study
BY/Seg5	Hybrid diploid BY4742/Seg5	this study
BY/Seg5 *she4Δ*	Hybrid diploid BY4742/Seg5 *SHE4^{BY4742} / she4^{Seg5}::KanMX*	this study
BY *she4Δ*/Seg5	Hybrid diploid BY4742/Seg5 *she4^{BY4742}::KanMX / SHE4^{Seg5}*	this study
BY/Seg5 *pep12Δ*	Hybrid diploid BY4742/Seg5 *PEP12^{BY4742} / pep12^{Seg5}::KanMX*	this study
BY *pep12Δ*/Seg5	Hybrid diploid BY4742/Seg5 *pep12^{BY4742}::KanMX / PEP12^{Seg5}*	this study
BY/Seg5 *cyc2Δ*	Hybrid diploid BY4742/Seg5 *CYC2^{BY4742} / cyc2^{Seg5}::KanMX*	this study
BY *cyc2Δ*/Seg5	Hybrid diploid BY4742/Seg5 *cyc2^{BY4742}::KanMX / CYC2^{Seg5}*	this study
V1116	Homothallic wine strain	Lallemand, Canada
V1116 *POX1*	V1116 *POX1^{V1116}::PGKprom - POX1^{Seg5} - CYC1term*	this study
V1116 *COX8*	V1116 *COX8^{V1116}::PGKprom - COX8^{Seg5} - CYC1term*	this study
V1116 *SNQ2*	V1116 *SNQ2^{V1116}::PGKprom - SNQ2^{Seg5} - CYC1term*	this study
V1116 *THI73*	V1116 *THI73^{V1116}::PGKprom - THI73^{Seg5} - CYC1term*	this study
V1116 *CYB5*	V1116 *CYB5^{V1116}::PGKprom - CYB5^{Seg5} - CYC1term*	this study
VR1	Natural isolate and former bioethanol production strain in Brazilian bioethanol production	Fermentec, Brazil
CBS1585	Heterothallic diploid sake strain, with high ethanol production capacity	CBS Utrecht, The Netherlands
FALI ES1	Bioethanol production	AB Mauri, Australia
FALI ES1 1X *AGT1*	Contains one active maltotriose transporter (*AGT1*$^{SAGY1403-7A}$)	P. Vandecruys
FALI ES1 2x *AGT1*	Contains two active maltotriose transporters (*AGT1*$^{SAGY1403-7A}$)	P. Vandecruys
M315	HDY.GUF5 + 3h mutagenesis in 3% EMS, diploid strain *Mat*	Demeke et al., 2013
GS1.11-26	HDY.GUF5 background, evolved for D-xylose fermentation, diploid strain *Mat /*	Demeke et al., 2013
GS1.11-26 *apj1ΔΔ*	GS1.11-26, double deletion of *APJ1,* diploid strain *Mat /*	M. Demeke
GSE16	Hybrid between GS1.11-26 and ER17	Demeke et al., 2013a
T18 *HAA1**	GSF767 background, point mutation inserted in both copies of *HAA1*	M. Stojijkovic
T18	GSF767 background, evolved for fermentation in lignocellulosic hydrolysate	Praj Industries, India
ER42A	Segregant 42A of Ethanol Red	M. Demeke
ER7A	Segregant 7A of Ethanol Red, *Mat*	Hubmann et al., 2013
ER18	Segregant 18 of Ethanol Red, *Mat**α***	Meijnen et al., 2016
ER18 *apj1*	*apj1::NatMX*	This study
ER18 *APJ1**	*APJ1::APJ1^{VR1-5B}*	This study
ER18 *ADE1*KIN3**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5}*	This study

ER18 *MKT1**	*MKT1::MKT1$^{VR1\text{-}5B}$*	this study
ER18 *VPS70**	*VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1*KIN3*MKT1**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} MKT1::MKT1$^{VR1\text{-}5B}$*	this study
ER18 *ADE1*KIN3*VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1*KIN3*apj1*	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} apj1::NatMX*	this study
ER18 *ADE1*KIN3*APJ1**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} APJ1::APJ1$^{VR1\text{-}5B}$*	this study
ER18 *MKT1* VPS70**	*MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *MKT1* APJ1**	*MKT1::MKT1$^{VR1\text{-}5B}$ APJ1::APJ1$^{VR1\text{-}5B}$*	this study
ER18 *MKT1* apj1*	*MKT1::MKT1$^{VR1\text{-}5B}$ apj1::NatMX*	this study
ER18 *VPS70* APJ1**	*VPS70::VPS70$^{VR1\text{-}5B}$ APJ1::APJ1$^{VR1\text{-}5B}$*	this study
ER18 *VPS70* apj1*	*VPS70::VPS70$^{VR1\text{-}5B}$ apj1::NatMX*	this study
ER18 *ADE1* KIN3* APJ1* MKT1**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} APJ1::APJ1$^{VR1\text{-}5B}$ MKT1::MKT1$^{VR1\text{-}5B}$*	this study
ER18 *ADE1* KIN3* APJ1* VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} APJ1::APJ1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *APJ1* MKT1* VPS70**	*APJ1::APJ1$^{VR1\text{-}5B}$ MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1* KIN3* apj1 MKT1**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} apj1::NatMX MKT1::MKT1$^{VR1\text{-}5B}$*	this study
ER18 *ADE1*KIN3*MKT1* VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1* KIN3* apj1 VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} apj1::NatMX VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *apj1 MKT1* VPS70**	*apj1::NatMX MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1* KIN3* APJ1* MKT1* VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} APJ1::APJ1$^{VR1\text{-}5B}$ MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study
ER18 *ADE1* KIN3* apj1 MKT1* VPS70**	*ADE1::ADE1^{Seg5} KIN3::KIN3^{Seg5} apj1::NatMX MKT1::MKT1$^{VR1\text{-}5B}$ VPS70::VPS70$^{VR1\text{-}5B}$*	this study

Table 9: Plasmids used in this study.

Plasmids	Description	Reference
pJET 1,2-attB-KanMX-attP	Amp^R, TEF_p-KanMX, removal of marker via PhiC31 integrase	Wallace-Salinas et al., 2014
pCR TOPO Blunt II B-NatMX-P	Kan^R, TEF_p-NatMX, removal of marker via PhiC31 integrase	SBO 2010
pBEVY-hph-PhiC31	Amp^R, TEF_p-HphMX, constitutive expression of the PhiC31 integrase	SBO 2010
pDONR201	Kan^R, gateway cloning site, donor vector (empty vector)	Invitrogen
pVV214-hph	Amp^R, *URA3* replaced by HphMX, 2µ, gateway cloning site, destination vector	Van Mullem et al., 2003
pVV214-POX1	pVV214 $PGK1_p$-$POX1^{Seg5}$	this study
pVV214-SNQ2	pVV214 $PGK1_p$-$SNQ2^{Seg5}$	this study
pVV214-COX8	pVV214 $PGK1_p$-$COX8^{Seg5}$	this study
pVV214-THI73	pVV214 $PGK1_p$-$THI73^{Seg5}$	this study
pVV214-CYB5	pVV214 $PGK1_p$-$CYB5^{Seg5}$	this study
pUG66	Amp^R, loxP-pAgTEF1-ble-tAgTEF1-loxP	Güldener et al., 2002
pUG6	Amp^R, loxP-pAgTEF1-KanMX-tAgTEF1-loxP	Güldener et al., 2002
pNAT-Cre	Amp^R, NatMX, galactose induced Cre recombinase expression vector	Hubmann et al., 2013
pgRNA-G2-HPH	Amp^R, TEF_p-HphMX, expression highly efficient guide RNA G2 (GGATGAGAATCTGACAAAGG)	B. Souffriau
pTOPO-A1-G2-B-KanMX-P-G2-A2	Amp^R, TEF_p-KanMX, scarless removal of marker mediated by Crispr/Cas9	B. Souffriau
pTOPO-A1-G2-B-NatMX-P-G2-A2	Amp^R, TEF_p-NatMX, scarless removal of marker mediated by Crispr/Cas9	B. Souffriau
pTEF-Cas9-BLE	Amp^R, TEF_p-ble, TEF_p-Cas9, Crispr mediated gene inactivation or replacement	B. Souffriau

Table 10: Primers used in this study.

Gene/block	Primer Nr	Sequence	Used for
ADE1-KIN3 A1	B-9709	GAATCATAAGCATTGCTTACAAAGAATACACATACGAAAT ATTAACGATAGTGGTCGGCTGGAGATCGG	deletion
ADE1-KIN3 A2	B-9710	AATTTCAATGAGAGTGCTATTTGATAATTGAATCAATTCTC TGTGTGAGTAGCCGTTATGGCGGGCATC	deletion
APJ1	A-8552	AGTACGGTACCACAGATGAAGTCCTGATTCAAGAGCAGC AGGCGCCAGCTGAAGCTTCGTACGC	deletion
APJ1	A-8553	CGTCGAATCGACGGTTGTTCCTTTTCCGAGAATCTCTTTCA TTAAGCATAGGCCACTAGTGGATCTG	deletion
APJ1 A1	B-1242	AAGTAAAACACATTTTTCTTTTGCTACCAGTGGACACAAG ATAACGGAGAGTGGTCGGCTGGAGATCGG	deletion
APJ1 A2	B-1243	AAAAAATAAAAATAGAAAATGTATAACATTACTGCATGAC TATATAACCTAGCCGTTATGGCGGGCATC	deletion
COX8 A1	B-321	TAGAGCCACAGAGCAAACTAAACAGAAAAGTTATCCATTT CCATTACGCAGTGGTCGGCTGGAGATCGG	deletion
COX8 A2	B-322	ACCAAGCACGCTATGTACACACATCTATCCTTCAAACTTAT GGGGGTGTTAGCCGTTATGGCGGGCATC	deletion
CYB5 A1	B-2943	CTTCATAGTAAAGAAAATCAATCAACTACAACATAAAATA TCAAACAATCGTGGTCGGCTGGAGATCGG	deletion
CYB5 A2	B-2944	AAAAATTCAATTGAGTTTGGAGAAGAGTGTGGGCTATTCT GGAAGAAAAAAGCCGTTATGGCGGGCATC	deletion
LEU2 A1	B-813	TTTTCTTACCTTTTACATTTCAGCAATATATATATATATATT TCAAGGATATACCATTCTAGTGGTCGGCTGGAGATCGG	deletion
LEU2 A2	B-809	TTCTATTATGAATTTCATTTATAAAGTTTATGTACAAATAT CATAAAAAAAGAGAATCTTTAGCCGTTATGGCGGGCATC	deletion
MKT1 A1	B-814	TTTTGAACTATAAAGTACTAAAGGCAGAAAAATTAATAGC AAATTAAGCGGTGGTCGGCTGGAGATCGG	deletion
MKT1 A2	B-810	GGGTGCTTTTTAAATAGTTCCACTATTTCCATCATACTCAT TCTCACGCTAGCCGTTATGGCGGGCATC	deletion
POX1 A1	B-317	CTAATAAGTATCACAGAAAAAAAGAAAATATAATAAATTA GTATTGCGATGTGGTCGGCTGGAGATCGG	deletion
POX1 A2	B-318	AAACAAAAGTCGCAAAACAGAGGGTTCGAAGGAAAACA GGAAACCTCTACAGCCGTTATGGCGGGCATC	deletion
SNQ2 A1	B-313	AAAAAAGTGGATAGAATAACACAGCTACCAAAATACGTA AAGAGAATTCAGTGGTCGGCTGGAGATCGG	deletion
SNQ2 A2	B-314	GAATTAAAGGCAGATGAATGCACAAAATGTTAAGTTATCT GAAGCCCACAAGCCGTTATGGCGGGCATC	deletion
THI73 A1	B-319	TTTACTTTGTTGTTGCTGAGAAGTAGTGAGAGTGTCAGCA AATCAATACAGTGGTCGGCTGGAGATCGG	deletion
THI73 A2	B-320	TGAATATTACAGACTAAAAATATTATATATTGTAAATTAAA AGTTGATTAAGCCGTTATGGCGGGCATC	deletion
VPS70 A1	B-1240	GTACTTATTAAAACAATTTCAACATGCACAATTAAGAATG TAAGAGATATGTGGTCGGCTGGAGATCGG	deletion
VPS70 A2	B-1241	ACGTATGCAAATAAAATAAATGTATATACTGAGGTGAGG TACTTAAATGCAGCCGTTATGGCGGGCATC	deletion
CYC2 A1	C-791	AAGAACCCTTCAAAAATAGAAAAGAGGAAACGAAAAAAA GAAAAAGTAGCGTGGTCGGCTGGAGATCGG	deletion RHA
CYC2 A2	C-792	CTACAGTATGTAATATTATTGTAATTGCCTTCTTAATCTCG AACGTTAAAAGCCGTTATGGCGGGCATC	deletion RHA
JHD2	A-8940	TGCCAAGTATTATTCTAAAAAATCATTACGCCATACACAA ATATTGAAGACGTACGCTGCAGGTCGAC	deletion RHA
JHD2	A-8941	AATTTTACCTCTAGATCATATTAACTAATCTCATCTTGCAC AAAAAACGTATCGATGAATTCGAGCTCG	deletion RHA
PEP12 A1	C-787	ATGCTTTTATAGAAGAATATAACGTAAATTACTACAATAA TTGTGTTGAGGTGGTCGGCTGGAGATCGG	deletion RHA
PEP12 A2	C-788	TAAGTGTATATATTATGTATATGATATTTGACGACGTGTG TTGGTTTGGTAGCCGTTATGGCGGGCATC	deletion RHA
RSF2	A-8948	AATTCTGTAAATACTATAGTATAGAGACGGCCGCCATTAT ATATTTGTAACGTACGCTGCAGGTCGAC	deletion RHA

RSF2	A-8949	AAGGTAACTGCGTATGCGGCATCGACAATAGGTAACTAC GCGAGCAACTTATCGATGAATTCGAGCTCG	deletion RHA
SHE4 A1	C-789	TTTTATCTAAACTACTCACATGAAAAGATTACTAAAAAATT AGAATCACGGTGGTCGGCTGGAGATCGG	deletion RHA
SHE4 A2	C-790	TATTTCTTAGTGAAAATTAAGAAGAGTTAAACATGGCGAT TCTTTCCATAAGCCGTTATGGCGGGCATC	deletion RHA
VPS70	A-8946	ACGTATGCAAATAAAATAAATGTATATACTGAGGTGAGG TACTTAAATGCCGTACGCTGCAGGTCGAC	deletion RHA
VPS70	A-8947	GTACTTATTAAAACAATTTCAACATGCACAATTAAGAATG TAAGAGATATATCGATGAATTCGAGCTCG	deletion RHA
YJR120W	A-8942	TACTCTAAACCAGTAATAAAGCCACATATATATGTATTTCC TTTCACGTGCGTACGCTGCAGGTCGAC	deletion RHA
YJR120W	A-8943	ACAAATTTGACTTGAGGAGGAGCACTTTTGTCTTCGTTTA TGAGGGGGGTATCGATGAATTCGAGCTCG	deletion RHA
YJR124C	A-8944	CTTGAAATGATCTACACATGTTTCTAAGTCACTCTACATTT GCTGCTGCACGTACGCTGCAGGTCGAC	deletion RHA
YJR124C	A-8945	AAAAGCTCATTGCAGAAGGATTTACCAATATTTCTAAAGT TAACGTAACCATCGATGAATTCGAGCTCG	deletion RHA
PTK2 A1	B-8801	GTTGTGTAAGCCGTAGCAGTTTTTTACAGGTTAACCGATT GAATAAGATAGTGGTCGGCTGGAGATCGG	deletion RHA
chrX block 1 (A1)	B-7711	ATCTATTACATTATGGGTGGTATGTTGGAATAAAAATCCA CTATCGTCTAGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 1 (A2)	B-7712	CTTATGTGTTTTAGTGTGTTTTCAAAAGGCATTTCTCTGCT CACGTTTAAAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 2 (A1)	B-7713	TTAGACATTTATGTAATTTTTAAGGCGCTAGTATAGATTAT TCAAAATTAGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 2 (A2)	B-7714	TGTTACATGCGTACACGCGTTTGTACAGAAAAAAAAGAA AAATTTGAAATAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 3 (A1)	B-7715	ACAGGCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTAT GTCACGCTTGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 3 (A2)	B-7716	AGGAATATTATAATAAATTAGACATTATAAATTAATAAGG TTACACGCATAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 3.1 (A2)	B-8794	GTCAATTTTCCGTTTTCTATCTAATACACGTTTTATATGTCT TTATTTTAAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 3.2 (A1)	B-8795	TAAAGAAAAAAGTTCGAAAACTTTTGTAGCAGTTGAAAGT TTTGTATGCTGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 3.2 (A2)	B-8800	AAAATGAATAAAATGCTCAATCTTAAAAATCATGCTAACA ACGATAATTCAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 3.3 (A1)	B-8799	GTAATAACTAGTTGAAGGTTCATATTATATAAAAAACTCT AAGAATAAAAGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 3.3 (A2)	B-8802	AATATGACGATACTAAAGCATAAAAGAGGGAATTAAAGA AGTCCTAACGTAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrX block 3.4 (A1)	B-8803	CAACATCAAGTGCTTAAAATATAATACGGTTTTCTACACTT TTATTAACGGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 4 (A1)	B-7717	AAAACGTTCCCTTATCCTTCGGAATAGGATAATAACAAAA ACCATTATTTGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrX
chrX block 4 (A2)	B-7718	GCATCTAGCGCACACAAACAACTACAAAACGCAATAACG ACACAGCAAGAAGCCGTTATGGCGGGCATC	deletion bulk RHA chrX
chrXV block 1 (A1)	B-8528	ACAACCATACAGAAATCAACGCGATTTCATCACACCATTC GCTTCTCTTCGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrXV
chrXV block 1 (A2)	B-8529	AAATAAAGGCAGCTGTACTTCTGCAGTTATGTCTTTTCGTC CACTGGGCCAGCCGTTATGGCGGGCATC	deletion bulk RHA chrXV
chrXV block 2 (A1)	B-8530	CGTCCCCCCTAAGAATAGCGAAGCGATATTACACTGAACA CTACAATGTCGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrXV
chrXV block 2 (A2)	B-8531	TTAAAAGACAGAGTTATAGTTTAGTAAATTTTCTTTTGAGT TAACGAGAGAGCCGTTATGGCGGGCATC	deletion bulk RHA chrXV
chrXV block 3 (A1)	B-8532	CACAACATCCCACATTACCCGATTGTTTGTTGCAAGATTTT TTGAAGAGGGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrXV
chrXV block 3 (A2)	B-8533	GGAAATCAGTGGTGGAAAAAGAATTGCCTTGCTAAGAGT ATTGTTGTCCAAGCCGTTATGGCGGGCATC	deletion bulk RHA chrXV
chrXV block 4 (A1)	B-8534	ATTGCAAACTCTATTTAACAACGAATGTAGATTATTATATG ACCAGAAAAGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrXV

chrXV block 4 (A2)	B-8535	GATAGATTGATTTTTTTAACCCATTTTACACATATCTTATG AAAGTGATAAGCCGTTATGGCGGGCATC	deletion bulk RHA chrXV
chrXV block 5 (A1)	B-8536	TATATTAAACAATAAGTATGAATGCCTAGTATAATTTCTTA GTTGTAGGAGTGGTCGGCTGGAGATCGG	deletion bulk RHA chrXV
chrXV block 5 (A2)	B-8537	TACTGAAATTTTTCAACAGAAGAAGTGCTTTTACTTCTCTC AATCTCTCCAGCCGTTATGGCGGGCATC	deletion bulk RHA chrXV
chrX SNP1	B-7738	CGATCTTGATCTCATCAGAC	allele specific PCR
chrX SNP1 (YJR030C) BY	B-7736	AAATATACTGCTCCTCCAGA	allele specific PCR
chrX SNP1 (YJR030C) Seg5	B-7737	AAATATACTGCTCCTCCAGC	allele specific PCR
chrX SNP2	B-7741	GAAGACTTGTAGGAAACCAG	allele specific PCR
chrX SNP2 (YJR039W) BY	B-7739	CTGATTCCAGAATGAGCACG	allele specific PCR
chrX SNP2 (YJR039W) Seg5	B-7740	CTGATTCCAGAATGAGCACC	allele specific PCR
chrX SNP3	B-7735	CGCTGCTATTGTAGGATAGC	allele specific PCR
chrX SNP3 (POL32) BY	B-7733	ATGGTATGAAGCTGTCCATG	allele specific PCR
chrX SNP3 (POL32) Seg5	B-7734	ATGGTATGAAGCTGTCCATC	allele specific PCR
chrX SNP4	B-7744	TCTCCACCTCTTTTTGGTCG	allele specific PCR
chrX SNP4 (OSM1) BY	B-7742	CTACAGCTTCTGAACTGGTA	allele specific PCR
chrX SNP4 (OSM1) Seg5	B-7743	CTACAGCTTCTGAACTGGTG	allele specific PCR
chrX SNP5	B-7616	ATCGAACCATCTCACCAACC	allele specific PCR
chrX SNP5 (PTK2) BY	B-7614	ACTTGTGTGACGACACAGA	allele specific PCR
chrX SNP5 (PTK2) Seg5	B-7615	ACTTGTGTGACGACACAGG	allele specific PCR
chrX SNP6	B-7622	ACGCAGCCAGAGCCAAAACT	allele specific PCR
chrX SNP6 (TOR1) BY	B-7620	ACTGCCAATCACCACTCCAT	allele specific PCR
chrX SNP6 (TOR1) Seg5	B-7621	ACTGCCAATCACCACTCCAC	allele specific PCR
chrX SNP7	B-8953	TCACTTGGGTTCTGGTTACC	allele specific PCR
chrX SNP7 (YJR084W) BY	B-8951	TTCTATGGATAGTGCGTCCA	allele specific PCR
chrX SNP7 (YJR084W) Seg5	B-8952	TTCTATGGATAGTGCGTCCG	allele specific PCR
chrX block 3.1	B-8956	GACAACCTGAAGTCTAGGTC	allele specific PCR
chrX block 3.1 SNP (UTR1) BY	B-8954	GTTGAGGTTGGAGTGCACAG	allele specific PCR
chrX block 3.1 SNP (UTR1) Seg5	B-8955	GTTGAGGTTGGAGTGCACAA	allele specific PCR
chrX block 3.2	B-8879	CTTGCACACTAAATCACTGC	allele specific PCR
chrX block 3.2 SNP (HIT1) BY	B-8877	GAAGCTAATGTGGAAGTTGT	allele specific PCR
chrX block 3.2 SNP (HIT1) Seg5	B-8878	GAAGCTAATGTGGAAGTTGC	allele specific PCR
chrX block 3.4	B-8882	TGTCTGCAAACGAGAACCAG	allele specific PCR
chrX block 3.4 SNP (YJR061w) BY	B-8880	AGGAAACTTGACGTTGAAGC	allele specific PCR
chrX block 3.4 SNP (YJR061w) Seg5	B-8881	AGGAAACTTGACGTTGAAGG	allele specific PCR
chrXV SNP1	B-8650	CAGGGCATACTTAGACACTG	allele specific PCR
chrXV SNP1 (AUS1) BY	B-8651	GCGTTGTTAAATCCACAAG	allele specific PCR
chrXV SNP1 (AUS1) Seg5	B-8652	GCGTTGTTAAATCCACAAC	allele specific PCR
chrXV SNP10	B-8663	CCATCCGCAACTTTACCTAC	allele specific PCR
chrXV SNP10 (SGO1) BY	B-8664	CCATCCGCAACTTTACCTAG	allele specific PCR
chrXV SNP10 (SGO1) Seg5	B-8665	CCAGAATCACCAAAGAACGC	allele specific PCR
chrXV SNP11	B-8502	GGTTGTCGTATTGGTCAAGG	allele specific PCR
chrXV SNP11 (TCB1) BY	B-8503	TTAGAGAACGCTGGTCAAGA	allele specific PCR
chrXV SNP11 (TCB1) Seg5	B-8504	TTAGAGAACGCTGGTCAAGG	allele specific PCR

chrXV SNP12	B-8666	ATCTAGTCCGTTATGTAGCG	allele specific PCR
chrXV SNP12 (PTC5) BY	B-8667	ATCTAGTCCGTTATGTAGCC	allele specific PCR
chrXV SNP12 (PTC5) Seg5	B-8668	ACGGTAGTGTTGAAGTTACC	allele specific PCR
chrXV SNP13	A-5591	CCGTATGGAAGAGGCCAAAG	allele specific PCR
chrXV SNP13 (RKI1) BY	B-8669	ACGGTAGTGTTGAAGTTACG	allele specific PCR
chrXV SNP13 (RKI1) Seg5	B-8670	CAAACTCTTCATCATCCTCG	allele specific PCR
chrXV SNP2	B-8653	GCGTCCAATTGAGATCTCAC	allele specific PCR
chrXV SNP2 (PET127) BY	B-8654	AGACGTCTATCTGTTGAGCC	allele specific PCR
chrXV SNP2 (PET127) Seg5	B-8655	AGACGTCTATCTGTTGAGCG	allele specific PCR
chrXV SNP3	B-8656	GTAGAACCACCAAACTGTGG	allele specific PCR
chrXV SNP3 BY	B-8657	GGACTGTGAAATTACACCTC	allele specific PCR
chrXV SNP3 Seg5	B-8675	GGACTGTGAAATTACACCTG	allele specific PCR
chrXV SNP4 (CIN5) BY	B-4925	CACCGGCCTTGCAATATGTG	allele specific PCR
chrXV SNP4 (CIN5) Seg5	B-4926	CACCGGCCTTGCAATATGTC	allele specific PCR
chrXV SNP4 CIN5	B-3604	GTAGATGCAGTAGTGTAGG	allele specific PCR
chrXV SNP5	C-835	GGGAAGTGTTGTTCAACAGC	allele specific PCR
chrXV SNP5 (PEP12) BY	B-8658	CCAATTGAGTGTTATCTGAG	allele specific PCR
chrXV SNP5 (PEP12) Seg5	B-8659	GACATAAAACTCCCTTTGG	allele specific PCR
chrXV SNP6	B-3603	CTCATCACTGGAACTTAGGG	allele specific PCR
chrXV SNP6 (STD1) BY	B-8505	TATTGCCTACTGCAGGAAC	allele specific PCR
chrXV SNP6 (STD1) Seg5	B-8495	TATTGCCTACTGCAGGAAG	allele specific PCR
chrXV SNP7	B-8660	TTCCTTCGACACTTGGTG	allele specific PCR
chrXV SNP7 (VHS3) BY	B-8661	TTCCTTCGACACTTGGTA	allele specific PCR
chrXV SNP7 (VHS3) Seg5	B-8662	TCACCAGAAATCCAAGACC	allele specific PCR
chrXV SNP8	B-8496	TATATCCTTGCAATCCAGCTCC	allele specific PCR
chrXV SNP8 (LPL1) BY	B-8497	AAATGCGCATGGCGTGATTGTG	allele specific PCR
chrXV SNP8 (LPL1) Seg5	B-8498	AAATGCGCATGGCGTGATTGTC	allele specific PCR
chrXV SNP9	B-8499	GGATCTTTGCCTTTCTCTCC	allele specific PCR
chrXV SNP9 (MSA1) BY	B-8500	GTTCGTTAGATGCAGTAGTCG	allele specific PCR
chrXV SNP9 (MSA1) Seg5	B-8501	GTTCGTTAGATGCAGTAGTCA	allele specific PCR
SHE4 SNP BY	C-831	TTGAAATGTCCGTTGAAGCG	allele specific PCR
SHE4 SNP Seg5	C-832	TTGAGATGTCCGTTGAAGCA	allele specific PCR
CYC2 SNP BY	C-833	CAATCTCTTTTGAAGCTGGG	allele specific PCR
CYC2 SNP Seg5	C-834	CAATCTCTTTTGAAGCTGGA	allele specific PCR
APJ1 SNP VR1-5B	C-100	CCATTAGAAGAGGGGGTAGC	allele specific PCR
APJ1 SNP ER/T18	C-101	CCATTAGAAGAGGGGGTAGT	allele specific PCR
KIN3 SNP ER18	C-634	CTGACTCAGTTCTCTTTCG	allele specific PCR
KIN3 SNP Seg5	C-635	CTGACTCAGTTCTCTTTCA	allele specific PCR
VPS70 SNP T18/S288c Rv	C-720	GTGATGCAAATAGTGGCAGC	allele specific PCR
VPS70 SNP VR1-5B	C-721	GTGATGCAAATAGTGGCAGT	allele specific PCR
VPS70 SNP VR1-5B	B-2335	TGTCATCGCGAGTGGTCTCA	allele specific PCR
VPS70 SNP S288c	B-2401	GTAGACACTAACGTAGCTCC	allele specific PCR
APJ1	A-7853	TGATGGGCACGGTGGTCTA	QPCR
APJ1	A-7854	TTGAATACCTTGCCCTTTGCA	QPCR
COX8 Fw	B-5509	GAAAGACACCTTACGCCTTATCTCA	QPCR
COX8 Rv	B-5510	GCAACAAATGGAACAGCAAATC	QPCR
CYB5 Fw	B-5505	CGAAGCATTGAGACTACTGAAAGG	QPCR
CYB5 Rv	B-5506	CCACAGAAACGCGCTCACT	QPCR
POX1 Fw	A-8022	TCCTCTGAGAGACCCTTCGACTT	QPCR

POX1 Rv	A-8023	TCGCTCCAATATCCCCTATGG	QPCR
SNQ2 Fw	A-8193	CGATACCCCGGCGGATA	QPCR
SNQ2 Rv	A-8194	CTGGCTCGCAGTGTCTCTTG	QPCR
THI73 Fw	B-5507	CCTCGTTGATGGTCGTTCGT	QPCR
THI73 Rv	B-5508	CAACCAACTGCACTCGAGGAT	QPCR
MKT1	B-802	TTTTTTGCGGCCGCTTCTAGCTTGTCAGCTCGAC	T4 cloning
MKT1	B-803	TTTTTTCCGCGGCAGCAGGTTGAAGTATTGG	T4 cloning
APJ1	B-1177	TTTTTTGCGGCCGCAGCGACTTAGACATGTCCC	T4 cloning
APJ1	B-1178	TTTTTTCCGCGGGAGATGGAACTAAGGGGTAG	T4 cloning
VPS70	B-1175	TTTTTTGCGGCCGCCATGGTTTGCAATCAAGAGC	T4 cloning
VPS70	B-1176	TTTTTTCCGCGGCACTACCTGCCTTTTCAGTG	T4 cloning
COX8 attB Fw	A-9146	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTGTGCCAACAGATGAT	Gateway cloning
COX8 attB Rv	A-9147	GGGGACCACTTTGTACAAGAAAGCTGGGTTACGCTATGTACACACATCTA	Gateway cloning
POX1 attB Fw	A-9333	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGCGATATGACGAGACGTAC	Gateway cloning
POX1 attB Rv	A-9334	GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGAAACCTCTACTCACTTG	Gateway cloning
SNQ2 attB Fw	A-9339	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCAATGAGCAATATCAAAAGC	Gateway cloning
SNQ2 attB Rv	A-9340	GGGGACCACTTTGTACAAGAAAGCTGGGTTATCTGAAGCCCACATTACTG	Gateway cloning
THI73 attB Fw	A-9327	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGTCAGCAAATCAATACAATG	Gateway cloning
THI73 attB Rv	A-9328	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGTAAATGTACCTGAAATTTCTG	Gateway cloning

Appendix

QTL9 chr. X	Chromosomal position (bp)
block 1	483663 - 510188
block 2	510136 - 526883
block 3	526715 - 553425
block 4	553369 - 572124
block 3.1	526715 - 533760
block 3.2	534026 - 539267
block 3.3	538771 - 548244
block 3.4	548758 - 553425

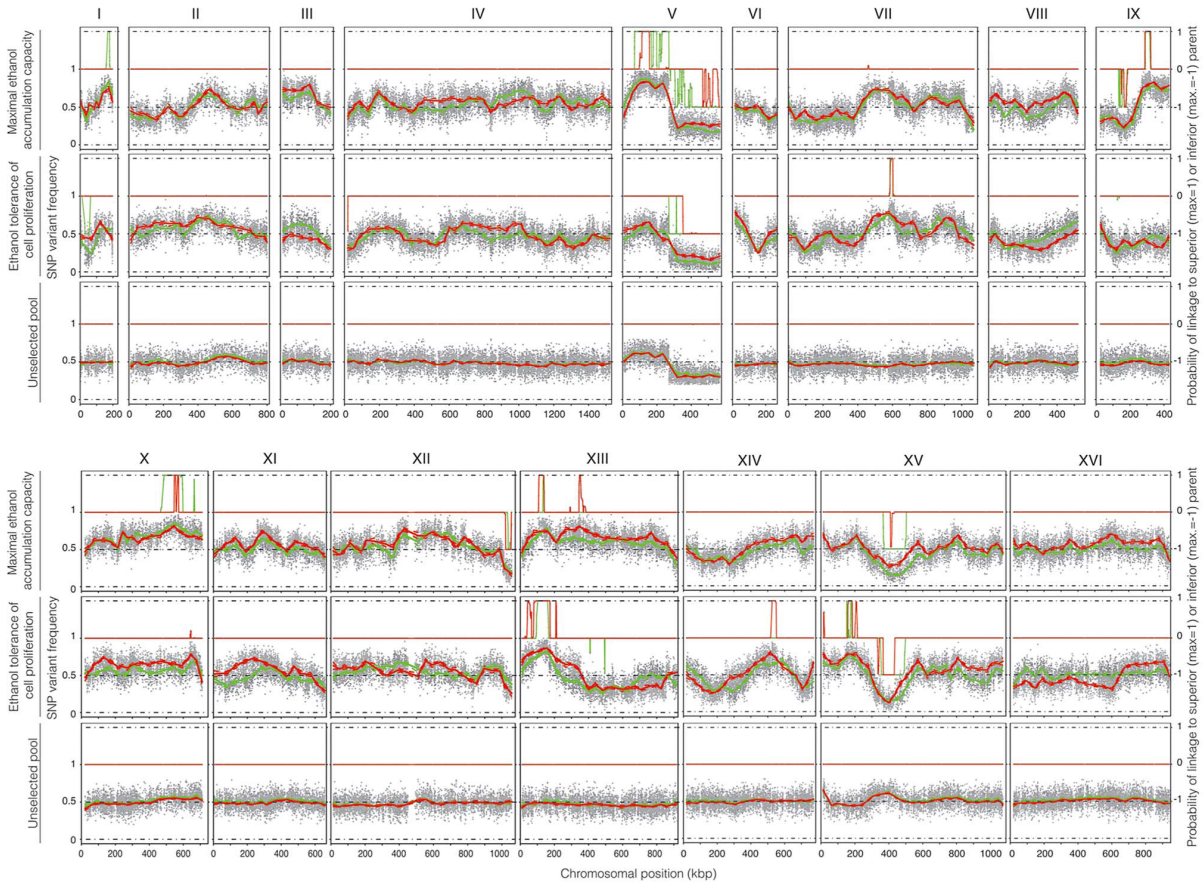
Appendix 1: Chromosomal position of the blocks and sub-blocks in QTL9 on chromosome X according to S288c reference genome (R64-1-1_20110203) Saccharomyces Genome Database

QTL17 chr. XV	Chromosomal position (bp)
block 1	364355 - 388688
block 2	389195 - 417841
block 3	418100 - 442554
block 4	442725 - 469252

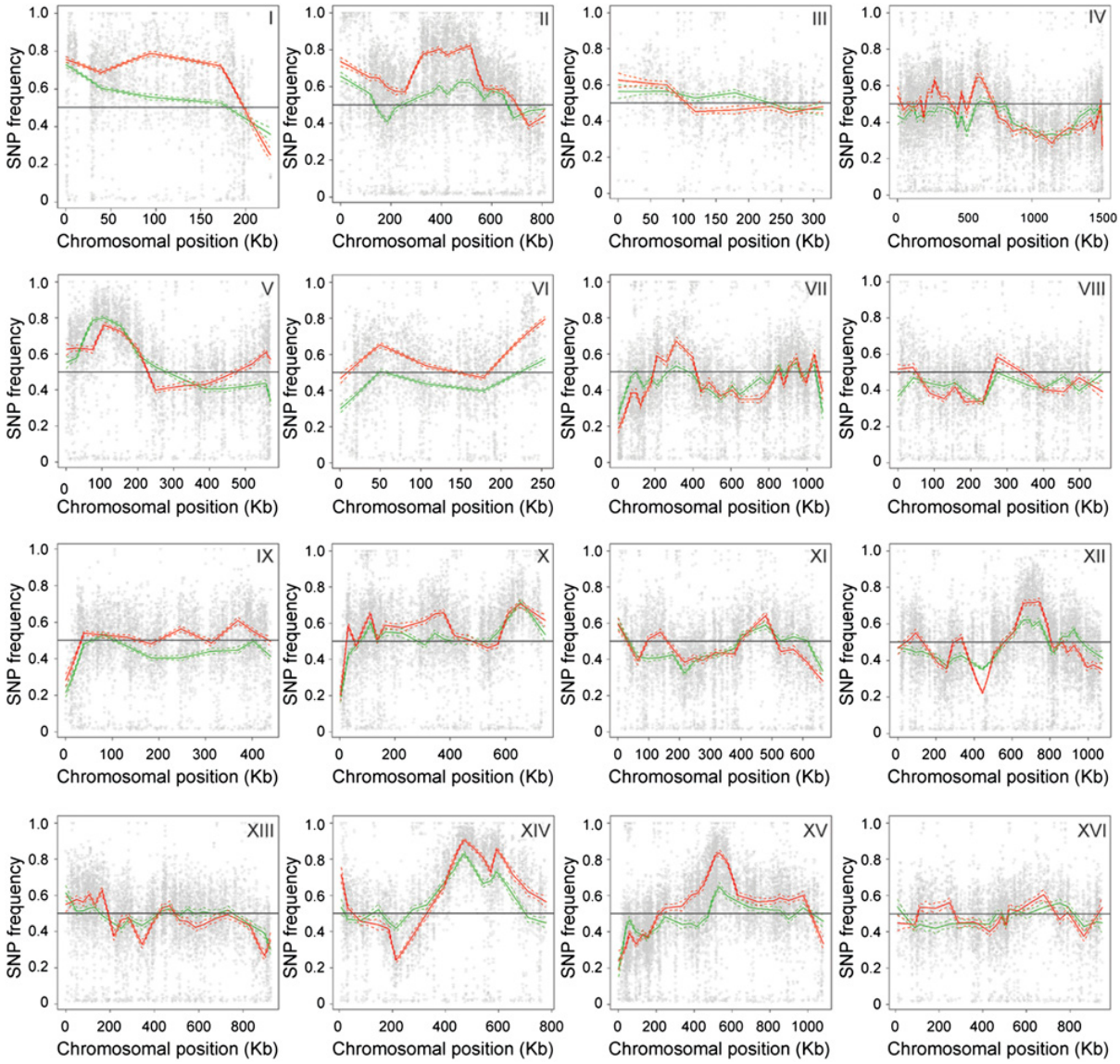
Appendix 2: Chromosomal position of the blocks in QTL17 in chromosome XV according to S288c reference genome (R64-1-1_20110203) Saccharomyces Genome Database

Appendix 3: Screening of 35 yeast strains containing an overexpressed candidate gene on plasmid pVV214 in small-scale fermentations in 100 mL YP + 33% (w/v) glucose. The strains are ordered in descending order as percentage of the control strain V1116-pVV214. The strains were evaluated once, twice (*) or three (**) times.

Strains	Relative ethanol production (% compared to V1116-pVV214)
V1116-plTHI73 (**)	107,8
V1116-plYMR175W-A (**)	106,9
V1116-plYBR056W-A (*)	105,7
V1116-plPOX1 (**)	105,3
V1116-plSNQ2 (*)	105,2
V1116-plTIR1 (**)	105,0
V1116-plCOX8 (**)	104,9
V1116-plEDC2 (**)	104,1
V1116-plKNH1 (*)	103,8
V1116-plYGL101W	103,8
V1116-plECM13	103,0
V1116-plCDC123 (*)	102,3
V1116-plYKR045C (*)	102,0
V1116-plPDR11 (*)	101,4
V1116-plCOX5a	101,3
V1116-plSIP18	101,3
V1116-plRMR1 (*)	101,2
V1116-plHPFLike (**)	101,1
V1116-plCYC1 (**)	100,9
V1116-plYDL114W (**)	100,8
V1116-plCYB5 (**)	100,8
V1116-plSPE2 (**)	100,8
V1116-plFMO1 (**)	100,7
V1116-plYOL159C (**)	100,4
V1116-plADH2 (**)	100,3
V1116-plSTB5 (**)	100,2
V1116-plYHR175W-A (**)	100,1
V1116-plTRM2 (**)	99,6
V1116-plHMG1 (**)	99,4
V1116-plYDL218W	98,8
V1116-plLCB5 (**)	98,2
V1116-plADE1	98,2
V1116-plKIN3 (**)	95,7
V1116-plRPI1 (*)	92,6
V1116-plROX1	91,7



Appendix 4: QTL mapping of maximal ethanol accumulation capacity (pool 1) and high ethanol tolerance of cell proliferation (pool 2). Whole genome sequencing analysis was performed by two independent companies utilizing the Illumina platform (BGI in green and GATC Biotech in red). An unselected pool (pool 3) was also sequenced twice to assess proper segregation of all chromosomes and possible linkage to inadvertently selected traits. The probability of linkage to the superior or the inferior parent, as determined with the HMM, is indicated on the right. Figure taken from Pais et al., 2013.



Appendix 5: QTL mapping of high ethanol tolerance by whole-genome sequence analysis. Pool 1 (green line) consist of 136 segregants tolerant to at least 16% v/v ethanol and pool 2 (green line) consist of 31 segregants tolerant to at least 17% v/v ethanol. The nucleotide frequency of quality-selected SNPs in the sequence of each pool was plotted against the chromosomal position. Three major QTL on chromosomes V, X, and XIV could be identified, and three minor loci in pool 2 on chromosome II, XII and XV. Figure taken from Swinnen et al., 2012.

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