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POR SARDEGNA
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Università degli Studi di Sassari
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RVRALIS ET SILVESTRIS SAPIENTIA

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Ciclo XXVI

A genome based approach to characterize genes involved in yeast adaptation to Sherry-like wines' biological ageing

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INDEX

Preface

Wine making is a very ancient process dating very likely from the beginning of agriculture and the most ancient remains of wine have characterized from pottery vessels found in Iran at Hajji Firuz Tepe (McGovern et al., 1996) dated between 5400-5000 BC. Close to this region the most ancient winery has been excavated in southeastern Armenia (Barnard et al., 2011). Since these very ancient times, wine production has changed drastically with the numerous innovations that lead to our modern wines. Indeed the first wines were kept in closed clays vessels, and were added resins, very likely as well as spices as preservatives (McGovern, 2003). These clay amphora were still porous to air leaving the possibility of wine to get oxidized.

Among these, three innovations had a great impact on wine styles. The use of sulphite is thought to be extremely early as Egyptians and Romans used sulphite to clean their wine containers (Romano and Suzzi, 1993). The use of wooden barrels (200 BC) and then of glass bottles in the middle of the 17th century with cork stoppers has provided another improvement of oxygen management in wine, that enabled wine ageing and paved the way to the modern wine style. Indeed, modern wine making processes try to limit the impact of oxygen in order to protect grapes and wine aromas. However, in contrast to this recent trend limiting the impact of oxygen, flor ageing developed in a few European countries (Hungary, France, Italy and Spain) appears as the opposite trend in which wine is deliberately (but moderately) oxidized by yeast in order to obtain the specific flavors and aromas of Vin Jaune (France), Vernaccia di Oristano (Italy), Tokay (Hungary) and Sherry wines (Spain). In addition these ageing methods produce wines almost stable towards oxidation. Interestingly Capece et al. (2013) reported recently a study of a wine making process performed in Georgia, the “Kakhetian” technique, in which fermentations take place in amphora in the presence of skins, peduncles, pips and stalks, and then take place a maceration phase. In this wine making process that can be considered as antique, flor yeast develop during the maceration/ageing final step. This suggests that flor/velum ageing is indeed a very ancient process and that ageing performed in Hungary, France, Italy and Spain are in fact remains of an ancient wine making process optimized for Sherry wine ageing.

Fermentation and flor ageing are performed by two different lineages of yeast strains (Legras et al., submitted), and the last decade has brought to light some of the specificities of flor yeast in comparison to wine, but these specificities still remain globally unknown.

In this thesis we have studied flor yeast in comparison to wine yeast in order to unravel their specificities. The genome sequence of 18 strains (wine and flor) from France, Hungary, Italy and Spain have been obtained. We have also developed a set of haploid flor strains for the molecular evaluation of different targets, and developed as well a synthetic media mimicking wine for that purpose. We present here the divergent regions that we have detected from the comparison of these genome sequences. For some of them, we have evaluated the impact of allelic variation in order to assess their role in the flor phenotype. The information gained by genome exploration of flor strains in comparison to wine strains should shed new light on the adaptation to flor ageing media and help to explain the genomic features peculiar to flor strains.

This manuscript presents first a bibliographic part where are illustrated different types of flor ageing process and the constraint they impose on yeast metabolism, the state of the art of biofilm build-up and *FLO11* regulation. Experimental data are detailed in three chapter: one presenting the construction of tools necessary for the evaluation of different target genes; another, consisting in a not yet published article, presenting the genome sequencing, the identification of regions that differentiate flor and wine strains and the evaluation of the allelic impact of some of them; and a third additional chapter describing the evaluation of some target genes for which we do not succeed in the identification of a differential phenotype. The experimental part is followed by a general discussion and conclusion.

1. INTRODUCTION

1.1 Comparison of Flor ageing processes

Flor ageing is performed in different countries, for Vin Jaune (France), Tokaji (Hungary), Vernaccia (Italy) and the most famous Sherry (Spain). Each vine area has developed a specific process of flor ageing.

The simplest ageing processes are performed in Italy (Sardinia) and France (Jura). In Sardinia, after the end of the fermentation the wine is transferred to chestnut and oak barrels left empty for 1/10 of the total volume, where biological ageing by flor strains takes place. Ageing process is then followed by an oxidative step (Del Caro et al., 2009). In Jura, alcoholic fermentation is performed separately in a cask or a tank, and after alcoholic fermentation, wines are transferred in another cask, where ageing takes place with or without inoculation with starter yeast (Levaux & Berthaud, 2011). These two systems are static (Fig. 1A) and inoculation is not always performed.

In contrast to Italian and French wines, Fino Sherry wine ageing are produced using a sophisticated system: the "sobretablas" and "solera" systems which alternate static and blending steps. "Sobretablas" is the first phase and consists in the fermentation of the must in 500-600 l oak casks to produce young wine that is fortified with 15-16% ethanol (v/v). In the second phase ("solera"), in which takes place the biological ageing of sherry wines, oak barrels are organized in rows that contain wine at different ageing stages: the lowest stage, called "solera", contains the oldest wine and is followed by stages, called first, second and third "criadera", containing wine progressively younger. Usually the system is a four-scale but sometimes the number of stages can reach up to 6. Commercial wine is collected from "soleras" and an identical amount corresponding to one third of the total volume is replaced with wine coming from the upper stage and so on till to replace the youngest wine at the top with that obtained from "sobretablas" (Fig. 1B) (Domecq, 1989). In this way there is a homogenization of the wine and the organoleptic profile is always similar inside oneself-scale. Some authors demonstrated that short and periodic aerations during the biological ageing or increasing the surface/volume ratio of the wine can reduce the length of this process (Mauricio and Ortega, 1997; Cortés et al., 1999; Saavedra and Garrido, 1961). The transfer of wine in the "solera" system, called blending, increases oxygen concentration in all the rows and diminishes the nutrient in a gradual way, from the upper "criadera" to the "solera". This leads to a progressive diminution of acetic acid,

glycerol and L-Proline while the concentration of acetaldehyde increases all along ageing (Berlanga et al., 2004).

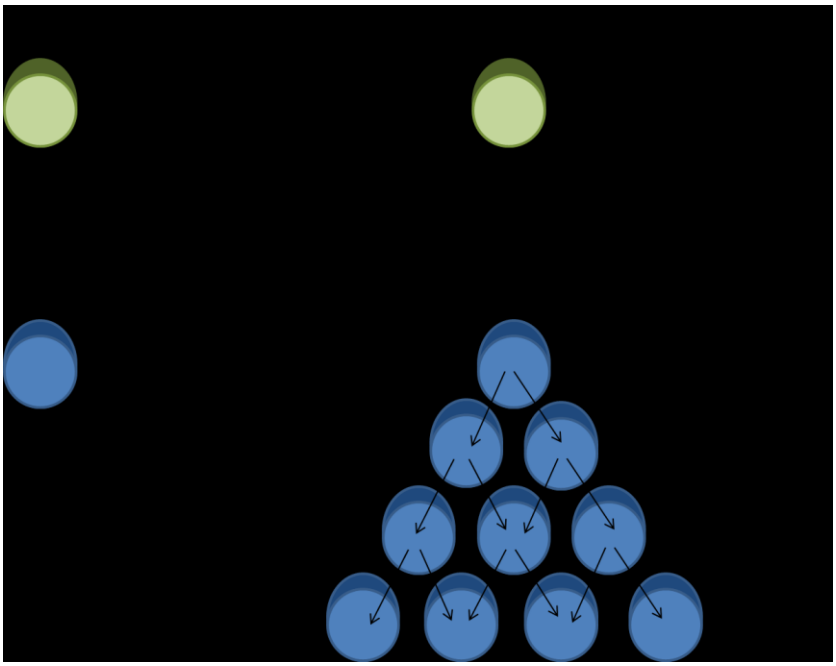


Figure 1: Sherry-like wine production process. Adapted image from Villamiel et al. (2008). A) Production system of Vin Jaune (France) and Vernaccia di Oristano (Italy). B) "Criadera/solera" system in Spanish Sherry production.

All these ageing processes share an interesting and surprising feature: the concentration of volatile compounds increased, which seems to be mainly due to water evaporation through the wood of the butts (Martínez de la Ossa et al., 1987).

1.2 Flor strains specificities

Flor strains have been found genetically isolated from wine strains (Sancho et al., 1986) and possess various distinctive genetic features that differentiate them from other wine strains.

A first genetic specificity of flor yeast has been reported in their ITS sequence: a 24-bp deletion in the *ITS1* region detected in Spanish flor strains and the insertion of a nucleic acid base C identified in the same region in French flor strains (Esteve-Zarzoso et al., 2001; Charpentier et al., 2009), leading to specific digestion profiles. Population studies have revealed a high mitochondrial DNA polymorphism (Esteve-Zarzoso et al., 2001; Castrejon et al., 2002) as well as large polymorphism in their karyotypes (Mesa et al., 1999).

More recently, the diversity of Jura flor yeast has been investigated with interdelta-typing and karyotyping, and reported a relation between flor strain genotype clusters and phenotypes (Charpentier et al., 2009). A more recent study compared the diversity of these Jura flor strains to strains from Hungary, Spain and Italy with microsatellite markers which revealed that all these yeast strains belong to the same genetic cluster (Legras et al., submitted).

The high karyotypic polymorphism of flor strains has been associated with aneuploidy and gross chromosomal rearrangements (GCRs) (Infante et al., 2003; Guijo et al., 1997). During a CGH-array comparison of two flor strains, Infante et al. (2003) found numerous duplicated genes, possibly provoked by the high frequency of double-strand breaks (DSBs) in DNA associated with the high acetaldehyde content produced during biological ageing (Ristow et al., 1995). They concluded that GCRs are an important factor for adaptive evolution of flor strains (Infante et al., 2003). Genes contained inside these duplicated regions may have a higher expression as a result of gene dose effect that could be useful for flor characteristics (Infante et al., 2003). Several recent experimental adaptation studies have shown that, indeed, aneuploidies can favor adaptation to harsh conditions (Dunham et al., 2002; Gresham et al., 2008, 2010; Infante et al., 2003).

However, recent CGH array results obtained on 6 flor strains isolated from Hungary, Spain, France and Italy, infirm the hypothesis that GCRs play a major role in the evolution of flor strains (Legras et al., submitted), as few duplicated genes were found in the genome of the strains being compared. In addition these authors reported ploidies close to 2n for most strains except for 2 Spanish strains. This suggests that the large aneuploidies observed by Infante et al. (2003) are rather more the results of the comparison of two strains of different ploidies than the results of the amplification of specific genes.

Flor strains are also characterized by poor sporulation and nonviable spores (Ibeas and Jiménez, 1996; Budroni et al., 2000) even though some differences can be observed between strains (Legras, personal communication), and by the incapacity to ferment galactose and maltose (Budroni et al., 2005). Low spore viability together with the consumption of galactose and maltose seems to be inversely correlated to flor-forming ability in Sardinian flor strains (Budroni et al., 2005).

Moreover, Sardinian flor strains were described to undergo a different life cycle than wine strains (Santa Maria and Vidal, 1970; Budroni et al., 2000; Pirino et al., 2004). This semi-

homothallic cycle is characterized by an atypical segregation, 2 non-mating and 2 mating spores, dominant *HO* and Ya (or Yα) in homozygosis at both silent loci HML and HMR.

During fermentation flor strains are more affected than wine strains by lack of lipids in the must, showing lower viability and sluggish fermentation activity as a result of decreased production of unsaturated fatty acids and ergosterol (Zara et al., 2009). This phenomenon does not appear to be linked to defects in the functionality of lipid biosynthesis, because the saturation of the culture medium with oxygen restores proper cell lipid composition. The authors suggest that flor strains require a large amount of oxygen during the early stages of fermentation in order to successfully complete the first oxygen-dependent steps in the lipids biosynthesis. Moreover, the addition of lipid supplements could reduce the adverse effects of the production of reactive oxygen species (ROS) and oxidative damage during fermentation (Landolfo et al., 2008; Landolfo et al., 2010).

But, finally, one of the most important traits of flor strains is their ability to build biofilm, essential for the biological ageing of Sherry-like wines.

1.3 Wine fermentation vs flor ageing

Wine fermentation and flor ageing are two processes that take place in different environments, where yeasts display different lifestyles. Flor ageing is an oxidative process and this oxidative metabolism is necessary to keep flor strains at the wine surface. Indeed flor petite mutants cannot form velum and are more susceptible to ethanol (Jiménez and Benítez, 1988).

A key difference between wine and flor yeast comes from the differences in growth style: during wine fermentation yeasts are in suspension in the vessel as a pelagic growth whereas during biological ageing of Sherry-like wines, flor strains form multicellular aggregates and rise at the wine surface forming a biofilm. The manner the biofilm installed depends of the type of ageing (Sherry, Vernaccia di Oristano or Vin Jaune). Biofilm formation can install immediately at the end of fermentation, when the concentrations of any fermentable carbon source is null or undetectable (Martinez *et al.*, 1997a) or it can happen later. In the case of Vernaccia di Oristano flor strains can start to form biofilm when the fermentation is still ongoing (Budroni, personal communication).

Yeast requires carbon and nitrogen sources, oligoelements and vitamins for growth. During wine fermentation grape must is rich in nutrients: it contains a high concentration of

glucose and fructose (ranging from 150 to 260 g/L in mature grape juice) (Ribéreau-Gayon et al., 1998) and assimilable nitrogen in variable content depending on the must (from 50 to more than 400 mg N/L) (Bely et al., 1990), sufficient to provide growth in most cases. On the contrary, during flor ageing on wine, glucose and fructose are almost completely depleted, and ethanol and various by-products (glycerol, organic acids, higher alcohols and their acetate) have been produced during alcoholic fermentation. In wines, ethanol is produced in concentrations up to 16%, and the concentration in glycerol, which is the most abundant by product after ethanol and CO_2, reaches 6-10 g/L. Glycerol synthesis takes place mainly during the growth phase and plays a major role during wine fermentation to reoxidize the NADH excess produced during biomass formation.

The exhaustion of glucose and fructose and the presence of oxygen impose flor yeast cells to shift from fermentative to oxidative (respiratory) metabolism. Both ethanol and glycerol, the most abundant carbon sources, can sustain flor yeast growth but glycerol enables a much better growth than ethanol (Zara et al., 2010). Flor yeasts can also consume organic acids (acetic, lactic, citric and succinic) as carbon sources.

Another important source for yeast is nitrogen not only to ensure cellular growth and metabolism but also because low levels of this compound can cause stuck and sluggish fermentations (Bely et al., 1990; Mauricio et al., 1995) and amino acids are the precursors of higher alcohols which take part to the composition of wine aroma (Henschke and Jiranek, 1994). The major nitrogen compounds in musts are ammonium, arginine and proline (Huang and Ough, 1991; Ough et al., 1991). During fermentation the first to be used is ammonium, that when abundantly present causes the nitrogen catabolite repression, preventing the use of other nitrogen compounds (Beltran et al., 2004; 2005). Even arginine and other amino acids are consumed while proline is used only to a limited extent during wine fermentation because the lack of molecular oxygen, which is essential for the activity of proline oxidase (Duteurtre et al., 1971; Mantachian, 1984), and the inhibition of proline permease by the above-cited nitrogen catabolite repression (Lasko and Brandriss, 1981; Horák and Ríhová, 1982). Instead, during biological aging the main nitrogen source becomes proline (Botella et al., 1990; Mauricio and Ortega, 1997).

The aerobic growth from available carbon and nitrogen resources leads to significant changes in the general composition of the wine and as well in organoleptic features. Flor yeast form mainly acetaldehyde but other volatile compounds such as acetic acid, acetoin,

2-3 butanediol and diacetyl are also produced. Ethanol is metabolized to acetaldehyde which provokes a decrease of ethanol content, and the high concentration of acetaldehyde, a chemically reactive compound, is one of the most important characteristics of Sherry wines (Domecq, 1989). Acetaldehyde gives rise to the production of lactones such as solerone and sotolon, and the latter is very important for the flavor of the French Sherry-like wine (Dubois et al., 1976; Guichard et al., 1992). The concentration of glycerol decreases during wine ageing.

Other families of chemical compounds are changed during ageing: a significant reduction of total acidity is observed and this decrease could be explained by the metabolism of flor strains which assimilate especially acetic acid (Berlanga et al., 2004), however main organic acids, except acetic acid, do not seem to enable velum growth when provided as the sole carbon source (Zara et al., 2010). This consumption of acetic acid leads to the reduction of volatile acidity.

1.4 Biofilm

During biological ageing *S. cerevisiae* flor strains grow aerobically at the surface of the wine forming multicellular aggregates. This aggregation leads to the build-up of a biofilm, also called velum, and allows cellular population to overcome stress conditions, like nitrogen and sugar depletion, after the end of fermentation.

Many other microorganisms than flor yeast are able to form biofilms, which are a widely encountered mode of growth for microorganisms. Some bacteria, fungi and archaea are able to form multicellular aggregates, and should be seen as the default mode of life of microorganisms. Most of microorganisms form pathogenic biofilms and 65-80% of human microbial infections derive from these biofilms that are difficult to eradicate by conventional antimicrobial therapy (Costerton et al., 1999; Donlan and Costerton, 2002). These biofilms can be defined as surface-associated microorganisms embedded in a matrix of extracellular polymeric substances (Hall-Stoodley et al., 2004). Inside the biofilm, coordinated chemical communication between cells enables biofilms to behave as multicellular entities (Costerton, 1995). This structure confers unique benefits to cells: resistance to antibiotics, defence from toxic compounds, higher retention of extracellular enzymes near the cell, resistance to desiccation and control of competition and predation (Quintero and Weimer, 1995; James et al., 1996; Costerton et al., 1995).

1.4.1 Archaeal biofilms

Biofilms formed by archaea are the less studied even if frequently detected in different environments (Kruger et al., 2008; Zhang et al., 2008). Archaea can coexist in biofilm with bacteria both in natural habitats and in human diseases (Hall-Stoodley et al., 2004).

Formation of biofilms in archaea is induced by different stress conditions: high metal and antibiotics concentrations, oxygen, extremes of temperature and high pH (Lapaglia and Hartzell, 1997).

In archaea flagella are responsible of cell-cell connections and adhesion to surfaces promoting biofilm formation (Näther et al., 2006).

Archaeoglobus fulgidus forms a biofilm very heterogeneous which contains proteins, polysaccharides and metals. The property to include metals in its biofilms can have applications in the detoxification or in the concentration of metals (Lapaglia and Hartzell, 1997).

1.4.2 Bacterial biofilms

Bacterial biofilms were the first to be observed and are the most studied. Anthony van Leeuwenhoek, towards the end of the XVII century, observed aggregation of bacteria in the plaque development of teeth and Pasteur (1864) noticed aggregated bacteria during acetic fermentation.

Biofilm enables cells to resist in a hostile environment and many infections that involved bacterial biofilms are hardly eradicated with available antimicrobials (Costerton et al., 1999). Bacterial biofilms can be both directly and indirectly responsible for human infections. The first case is that of infective endocarditis (Donlan and Costerton, 2002), cystic fibrosis pneumonia (Koch and Hoiby, 1993; Lyczak et al., 2002) and dental caries while the second is due to contamination of medical devices like prostheses, catheters, cardiac pacemakers, endotracheal tubes, … (Hall-Stoodley et al., 2004).

Bacteria can form two biofilm structures: a heterogeneous mushroom-shaped during growth on glucose or a flat biofilm during growth on citrate (Klausen et al., 2003a).

Formation of the heterogeneous mushroom-shaped biofilm involves three structures: flagella, type I and type IV pili. Flagella give to cells the motility necessary to reach the surface overcoming its repulsive forces during the first steps of velum formation (O'Toole and Kolter, 1998a,b; Pratt and Kolter, 1998). Type I pili, which contain mannose-sensitive

adhesins, are necessary in the initial stages of biofilm formation and operate adhering to abiotic surfaces, carrying out a stable cell-surface attachment (Pratt and Kolter, 1998; Davey and O'toole, 2000). Then, motility and type IV pili help biofilm diffusion on the surface (O'Toole and Kolter, 1998a,b). In particular, type IV pili, during the last stages of biofilm formation, are used to climb up on the initial microcolonies formed making a cap and completing the biofilm mushroom-shaped (Klausen et al., 2003b).
In flat biofilm, instead, attachment of cells is independent from flagella and type IV pili, microcolony formation is due to clonal growth and biofilm diffusion is a consequence of twitching mobility (Klausen et al., 2003a).

1.4.3 Fungal biofilms

Like bacteria, some pathogen fungi are able to form biofilm on prostheses or catheters causing infections in patients (Kojic and Darouiche, 2004). Fungal biofilms can also be formed on industrial installations, having an important economic impact for food-processing companies (Verstrepen and Klis, 2006), and on biotic surfaces like oral mucosal (Dongari-Bagtzoglou et al., 2009).
Biofilm structure (cell-to-cell aggregation, the presence of an extracellular matrix, metabolic plasticity and the up-regulation of efflux pump genes) provide a higher resistance towards host immune factors and antifungal drugs than planktonic cells (Fanning and Mitchell, 2012).

Biofilm formation in fungi is the result of three consecutive stages: adherence to the substrate, cell proliferation on the surface and formation of hyphae in the upper part of the biofilm (Finkel and Mitchell, 2011). Hyphae and biofilm formation is contact sensing: yeast cells begin to form hyphae and biofilm after contact with a surface (Kumamoto and Vinces, 2005). In a similar manner, some diploid strains of *S. cerevisiae* form pseudohyphae in response to the contact with a surface during nutrient deprivation (Gimeno et al., 1992; Lorenz et al., 2000). *Candida albicans* produces a different response in function of the type of surface with which takes contact: biofilm formation or filamentation occurs after contact with a solid or a semisolid surface respectively (Kumamoto, 2005). For pathogenic fungi contact sensing is important for virulence: the contact with a surface induces formation of invasive hyphae that allow the fungus to reach the bloodstream favouring the onset of infections (Kumamoto, 2008).

Some pathogenic fungi, like *C. albicans* and *Cryptococcus neoformans*, are able to carry out dimorphic transition switching from the yeast form to the more invasive hyphal form (Sudbery et al, 2004). Both are important for virulence, hyphae allowing invasion while yeast form being the first responsible of dissemination of infections (Jacobsen et al., 2012; Saville et al., 2003). A large number of environmental signals can cause morphogenesis: temperature, pH, CO_2, different carbon sources, Ca^{2+}, proline, N-acetylglucosamine, low ammonia and others (reviewed in Han et al., 2011). The switching in *C. albicans* is mainly regulated by two pathways activated by external stimuli: the Mitogen-Activated Protein Kinase (MAPK) pathway and the cAMP-Protein kinase A (PKA) pathway (Han et al., 2011).

1.5 Quorum sensing

Formation of all biofilm is mediated by mechanisms of *quorum sensing*. In a biofilm cells are interactive organisms belonging to a microbial community that manifest coordinate group behaviours. This phenomenon is activated through the production of autoinducers, small molecules that allow cell-to-cell communication (Nadell et al., 2008). These molecules are released by cells and are accumulated depending on cell density. When *quorum* (population density threshold) is reached autoinducers interact with receptors, which control gene expression, on the cell surface. In this way takes place *quorum sensing*, the coordinated gene expression obtained in function of population density (Fuqua et al., 1994). *Quorum sensing* is responsible of different effects in cells such as antibiotic biosynthesis (Bainton et al., 1992) or dimorphic transition (Hornby et al., 2001), and biofilm formation (Alem et al., 2006).

For *C. albicans*, quorum sensing can be obtained through the action of aromatic alcohols and farnesol (Hornby et al., 2001). Farnesol can block biofilm formation (Langford et al., 2009). In *S. cerevisiae* the aromatic alcohols, phenylethanol, tryptophol, have been described to act as autoinducers in filamentous growth (Chen and Fink, 2006). Each of these alcohols triggers similar but slightly different responses under the control of Cat8 and Mig1 (Wuster and Babu, 2010). The production of autoinducers can be affected by availability of their precursors, such as aromatic amino acids in *C. albicans* (Ghosh et al., 2008).

1.6 Extracellular matrix

Biofilm formation begins with the interaction of cells with a surface, afterwards the production of extracellular matrix contributes to the architecture of the velum (Davey and O'toole, 2000). Most biofilms present a variable and complex matrix structure: cells embedded in it can form a thin and dispersed layer or a thick one, different sections of matrix display changes in density and channels are present allowing the passage of nutrients and metabolites (Sutherland, 2001). Due to the variety of its constituents, such as water, exopolysaccharide (EPS), cell components derived from cell lysis and substances coming from the surrounding environment, the matrix contributes to the creation of a heterogeneous environment (Davey and O'toole, 2000; Sutherland, 2001). The nature of the matrix is dependent on intrinsic and extrinsic factors: the first comprise genetic features of microorganisms, the second is due to physico-chemical characteristics of the environment (Wimpenny, 2000; Sutherland, 2001). EPS, one of the matrix components, has been reported to have benefits. In fact, it can hamper the access of some antimicrobial agents into the velum (Gilbert et al., 1997), sequester cations, metals and toxins (Decho, 1990; Flemming, 1993) and protect from different environmental stresses, such as osmotic shock, UV radiation, pH changes and desiccation (Flemming, 1993). The amount of EPS is variable depending on the microorganism and increases as the biofilm gets older (Davey and O'Toole, 2000; Varon and Choder, 2000; Branda et al., 2005).

In some strains, *C. neoformans* and *Candida* spp., the extracellular matrix is also important for pathogenicity (Mukherjee et al., 2005; Jain et al., 2006).

Most of the information available on the extracellular matrix concerns bacteria and fungi, little is known about *S. cerevisiae*. The presence of an extracellular matrix was observed in the air-liquid interfacial biofilm of Sardinian flor strains but till now nothing is known about its composition (Zara et al., 2009).

1.7 Flor yeast velum and role of *FLO11*

Gaining the ability of adhere to each other is a crucial point for the build-up of a biofilm. This ability of yeast cell is obtained through the activation of different adhesins which have been identified in different yeast strains. They include *FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11* which are cell wall-associated surface protein (Brückner and Mösch, 2012) *FLO1*, *FLO5*, *FLO9* act as lectins, that recognize mannose oligomers using a Ca^{2+} binding motif

whereas *FLO10* and *FLO11* are able to confer cell-to-cell adhesion in a mannose and Ca^{2+} independent manner (Brückner and Mösch, 2012). The activation of *FLO10* and *FLO11* lead to an increased cell surface hydrophobicity.

Cells lipid content was found elevated and/or altered in biofilm (Farris et al., 1993; Iimura et al., 1980a, 1980b; Martínez et al., 1997), but several authors individuate in *FLO11* the gene responsible of the increased hydrophobicity during biofilm formation (Fidalgo et al., 2006). This gene, localized on the IX chromosome, consists in a 4104 bp ORF (Lo and Dranginis, 1996) and its promoting region contains 4 upstream activation sequences (UASs) and 9 repression elements, reaching a length of at least 2800 bp (Rupp et al., 1999). This gene encodes a glycoprotein localized in the cell wall and contains three domains: a C-terminal domain containing the consensus sequence for the GPI anchor, a central domain containing highly repeated sequences rich in serine and threonine and a hydrophobic N-terminal domain (Lambrechts et al., 1996; Lo and Dranginis, 1996). These domains are retained to be essential for the floatability of flor strains. In fact, the C-term could anchor the glycoprotein to the membrane and the central domain could promote the elongation of the protein across the cell wall exposing the hydrophobic N-terminal domain on the cell surface (Teunissen et al., 1995; Fidalgo et al., 2006).

The higher hydrophobicity of the Flo11 protein seems due to the expansion of the central domain (Reynolds and Fink, 2001; Zara et al., 2005; Fidalgo et al., 2006) which is glycosylated. The glycosylation of this central core of the adhesion is essential for adhesion (Meem et al., 2012). In particular, mutations in the promoter and in the coding region of the gene *FLO11* are responsible of the acquisition of floatability: a deletion of 111bp in the promoter which comprises a repression sequence necessary to inhibit *FLO11* that results in higher expression levels and a minisatellite expansion which amplification corresponds to an increased hydrophobicity (Fidalgo et al., 2006). A study carried out on 20 Sardinian wild flor strains revealed that *FLO11* is highly polymorphic and that the ability to produce biofilm is dependent not only on the expansion of the repeated sequences but also on the transcriptional levels of the gene (Zara et al., 2009). Additional analysis made among Jura flor strains indicated that the 111bp deletion in the promoting regions was sometime missing but was accompanied by an increase in the length of *FLO11* (Legras et al., submitted).

Zara et al. (2005) proposed a model for the air-liquid interfacial biofilm formation based on the increased surface hydrophobicity due to the glycoprotein Flo11: at the diauxic shift the *FLO11* expression increase results in a higher cell surface hydrophobicity, in line with the formation of multicellular aggregates that capture bubbles of CO_2 coming for fermentation of residual sugar allowing the floatability of aggregates and velum formation.

Even though Flo11 has been shown to be the main player in biofilm formation, but one can wonder if other parietal proteins contribute to a lower extent to biofilm formation, in this sense, the contribution of Flo1 and Flo10 which are also involved in cellular adhesion phenotypes (Bester et al., 2012) has to be more carefully evaluated. Furthermore, Flo10 has the same structure of Flo11 and it will be not surprising that Flo10 also has a role in biofilm formation. In addition two other proteins were described to have a role in its building up. A 49-KDa mannoprotein was found contributing to cell surface hydrophobicity and probably in biofilm formation in French flor strains (Alexandre et al., 2000) and a point mutation or deletion of *HSP12*, which encodes a heat-shock protein, leads to a failure in velum formation in Sardinian flor strains (Zara et al., 2001).

1.8 *FLO11* regulation

Much of *FLO11* regulation has been gained from the study of pseudohyphal growth, which revealed that its regulation is one of the most complex of *S. cerevisiae* genome. The expression of *FLO11* is finely tuned with environmental response such as nutrient deprivation (nitrogen, and sugar), pH and stress. This complex regulation results from the combination of 3 main pathways: the filamentous growth MAPK pathway, the Ras/cAMP/PKA pathway, and the glucose repression pathway. But three other regulatory pathways have also been described: the "Target of Rapamycin" (TOR) pathway, a pH sensing pathway and an epistatic pathway (Verstrepen and Klis, 2006; Cullen and Sprague, 2012). *FLO11* expression is triggered by two long intergenic noncoding RNAs (ncRNAs) (Bumgarner et al., 2009) on which acts the different regulatory transcription factors, and is also under epigenetic control (Frieman and Cormack, 2004; Halme et al., 2004; Barrales et al., 2012). Nutrient depletion or stress factors activates its transcription (Sampermans et al., 2005) while availability of glucose represses it.

1.8.1 Filamentous growth MAPK pathway

The filamentous growth MAPK pathway (FG pathway) is activated by nutrient deprivation (Fig. 2). Msb2, a signaling mucin, is believed to be a receptor for the external signals that activate this pathway and might act in response to stress or nutrient deprivation (Cullen et al., 2004; Cullen and Sprague, 2012). Msb2 associates with the transmembrane osmosensor Sho1 to transmit the signal to the downstream components of the pathway (Tatebayashi et al., 2007). Cullen et al. (2004; 2012) proposed a model where Msb2 activated by the signal and associated with Sho1 bring the guanidine nucleotide exchange factor (GEF) Cdc24 in proximity of its Rho-like GTPase Cdc42, which is then activated and can bind and transmit signal to its effector proteins, among which the MAPKKK Ste20. Then, GTPase-activating proteins (GAPs) Rga1 and Rga2 can turn back Cdc42 to its inactive form Cdc42-GDP, accelerating GTPase activity (Smith et al., 2002; Tcherkezian and Lamarche-Vane, 2007). Ste20 activates the MAPK cascade phosphorylating Ste11, which forms a complex with Ste50 and in turn phosphorylate Ste7. The MAPK/ERK (Extracellular signal Regulated) kinase Ste7 then phosphorylates Kss1 (Madhani and Fink, 1997). Kss1 triggers filamentous growth in two opposed different manners: it activates filamentation through its kinase-dependent activity and inhibits it as well through a kinase-independent role (Madhani and Fink, 1997). When the FG pathway is activated Ste7 phosphorylates Kss1, which becomes an activator. Kss1 shows its kinase activity phosphorylating Ste12 (Madhani and Fink, 1997). In this way, Ste12 together with Tec1 binds at filamentous growth response elements (FRE) in the promoter of *FLO11* and triggers filamentation (Lo and Dranginis, 1998; Rupp et al., 1999; Gagiano et al., 2002). Another player, Mss11 acts as a transcriptional activator of *FLO11* (van Dyk et al., 2005) which interacts with Ste12 and Tec1. The repressive kinase-independent role, instead, takes place in absence of stimuli for filamentous growth, and in this case Kss1 binds to Ste12 and inhibits its function of transcriptional activator of *FLO11* (Lambrechts et al., 1996; Bardwell et al., 1998). The two nuclear proteins, Dig1 and Dig2, bind to Kss1 and Ste12 during repression (Cook et al., 1996; Tedford et al., 1997). Kss1, when activated, phosphorylates Dig1, Dig2 and Ste12 allowing a full derepression (Bardwell et al., 1998).

Recent data indicate also that nitrogen transport affects pseudohyphal growth. Rutherford and collegues (2008) reported first that the ammonium permease Mep2 transmits the signal along the MAPK pathway. However, more recently Torbensen et al. (2012) reported that

amino acid transporters *GAP1*, *DIP5* and *GNP1* are essential for biofilm formation. Deletion of *GAP1* induced a MAP kinase, cAMP/PKA, *FLO11* independent invasive growth. Meanwhile, Judeh et al. (2013) inferred from microarray data obtained under nitrogen stress that the deletion of *FLO8* is likely to affect the biosynthesis/metabolism of sulfur containing nitrogenous compound and impair transport of nitrogen compounds (Judeh et al., 2013).

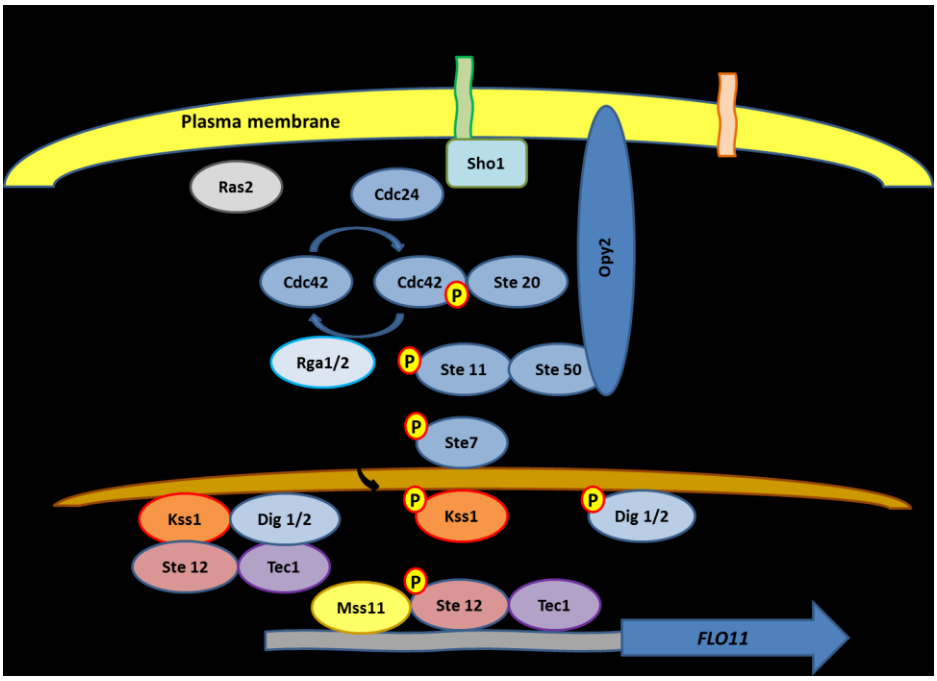


Figure 2: Filamentous growth MAPK pathway. Image adapted from Gagiano et al. (2002), Verstrepen and Klis (2006). Some associations are not shown to simplify the picture.

Several component of the pathway are associated in complexes: Msb2 associated not only with Sho1 but also with Cdc42 (Cullen et al., 2004), Opy2 associates with the adaptor Ste50 (Wu et al., 2006; Ekiel et al., 2009) which, in turn, associates with Cdc42 and Ste11 (Posas et al., 1998; Truckses et al., 2006; Garcia et al., 2009), Cdc42 binds also Ste20 (Simon et al., 1995; Zhao et al., 1995; Peter et al., 1996), inactive Kss1 associates with Dig1, Dig2, Ste12 and Tec1 (Cook et al., 1996; Tedford et al., 1997; Gagiano et al., 2002), phosphorylated Ste12 binds Tec1 in the promoting region of *FLO11* (Madhani and Fink, 1997).

In addition to Cdc42 another GTP-binding protein, Ras2, has been proposed to transmit the signal along the FG pathway. Some authors have suggested that Ras2 acts upstream of Cdc42 (Mösch et al., 1996) but is not clear how it can regulate the pathway. To date the involvement of Ras2 in the FG MAPK pathway cannot be excluded but there is no evidence of its role in this pathway. In fact, a direct association with the upstream

components of the pathway has been excluded (Cullen and Sprague, 2012): Msb2 binds Cdc42 but not Ras2 (Cullen et al., 2004), Ste50 links to Cdc42 but not Ras2 (Truckses et al., 2006).

Some of the components of the filamentous growth MAPK pathway (Cdc42, Ste20, Ste11, Ste50 are also shared by other two MAPK pathways (Fig. 3): the mating pathway and the High Osmolarity Glycerol (HOG) response pathway.

The mating pathway is activated by pheromone, which is sensed by Ste2/Ste3 receptors. Ste2 is present in MATa cells, Ste3 in MATα cells (Jenness et al., 1983; Hagen et al., 1986). When pheromone signal reaches cell wall, the receptor changes conformation, transmitting the signal to the G protein with which it is associated in the plasma membrane and activates the downstream components of the pathway (Konopka and Fields, 1992). As the FG pathway, the complex Cdc42/Ste20 phosphorylates Ste11 activating the MAPK cascade, but unlike the FG pathway the last kinase is not Kss1 but Fus3 (Elion et al., 1991; Cullen and Sprague, 2012), which phosphorylates Ste12 (Elion et al., 1993). The transcription factor Ste12 is also present in the FG pathway and it has been proposed that signalling specificity is due to Tec1, another transcription factor that acts as a coregulator during filamentous growth while it is degraded during mating (Madhani and Fink, 1997; Bao et al., 2004; Cullen and Sprague, 2012). Ste5 a large scaffold protein that associates with Fus3, Ste7, Ste11 is a component of the mating pathway not shared by the other two MAPK pathways (Cullen and Sprague, 2012) and the β subunit of the G protein (Whiteway et al., 1995). The β subunit associates also with Ste20 (Leeuw et al., 1998).

The HOG pathway is activated by high extracellular osmolarity and the signal is sensed by 2 membrane sensor proteins that transmit the osmotic stimulus along 2 separated branches: Sho1, osmosensor present also in the FG pathway, and Sln1 (Maeda et al., 1994, 1995). Sho1 associates with two signaling mucins, Msb2 and Hkr1, to transmit the signal (Tatebayashi et al., 2007). The downstream components from Sho1 to Ste11 are shared by the FG pathway. When activated, Ste11 associates and activates Pbs2, MAPKK and scaffold protein common to both branches of the HOG pathway (Cullen and Sprague, 2012). When the osmotic stimulus reaches Sln1, its histidine kinase activity is repressed, unphosphorylated Ssk1 accumulates inducing the autophosphorylation of Ssk2/22 that phosphorylate Pbs2 (Maeda et al., 1994; Posas et al., 1996; Posas and Saito, 1998). Pbs2, when activated, phosphorylates the MAPK Hog1, which in turn phosphorylates different

transcription factors (Westfall et al., 2004). Thus, both branches sense hyperosmolarity but transmit the signal to Pbs2 independently of each other.

The mating and the HOG pathway not only share some of the components with the FG pathway but can also regulate it. Hog1 inhibits the filamentation pathway and it has been proposed that it could act by phosphorylating and, hence, inactivating a component of the pathway, but is still unclear which component of the FG pathway is the target of Hog1 (Cullen and Sprague, 2012). Also Fus3 has been proposed to have an inhibitory function in filamentation and the mating pathway can regulate the transcription of Tec1 and Kss1 (Oehlen and Cross, 1998; Roberts et al., 2000). Moreover, Kss1 is partially activated in response to pheromone and osmotic stress (Cullen and Sprague, 2012).

Cappell and Dohlman (2011) reported that *PIK1*, encoding Phosphatidylinositol 4-kinase (PtdIns 4-kinase), among its different roles inside the cell has also the function to regulate the activity of these three MAPK pathways in yeast. Pik1 carries out its regulatory activity promoting the activation of Fus3 and Hog1, MAP kinases that induce respectively mating program and high osmolarity glycerol response, and repressing the activation of Kss1, MAP kinase which, when active, induces filamentous growth. This regulation is achieved, very likely in an indirect way, through upstream components common to all three pathways: Ste11, Ste50 and Opy2 (Cappell and Dohlman, 2011). Thus, *PIK1* contributes to filamentous growth but its involvement in the regulation of biofilm formation is not known.

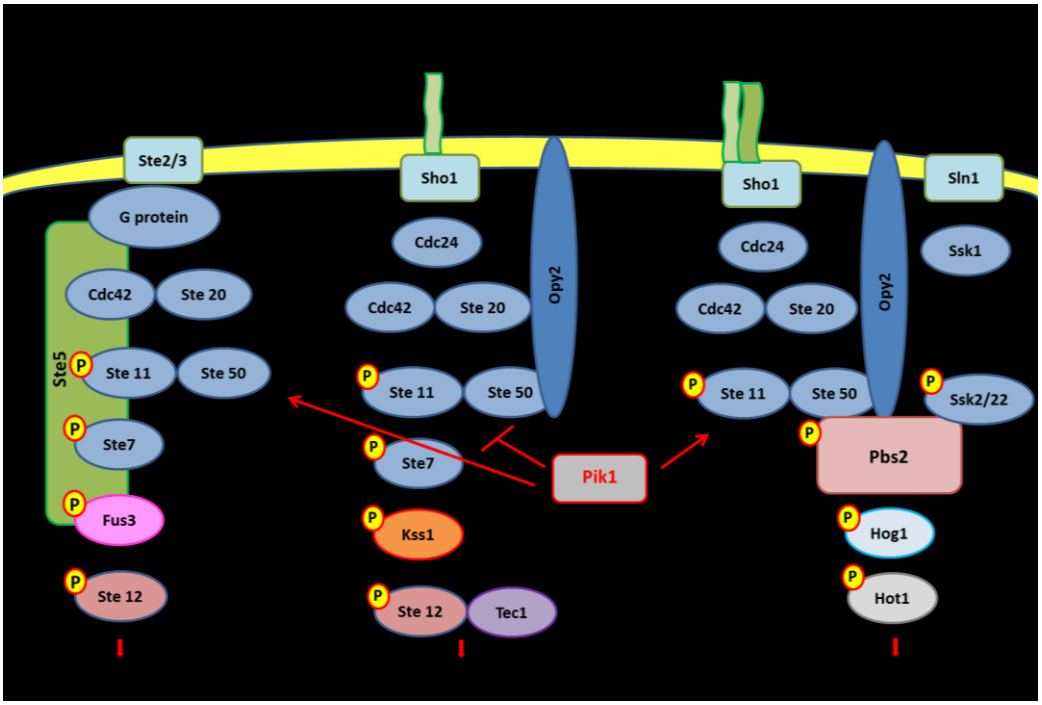


Figure 3: Schematic representation of Mating, Filamentous growth and HOG MAPK pathways. Image adapted from Cappell and Dohlman (2011), Cullen and Sprague (2012).

1.8.2 cAMP/PKA pathway

This pathway can be activated independently via Ras2 or Gpr1/Gpa2 (Fig. 4). In response to a glucose signal the small G protein Ras2 binds its GEF, Cdc25, which induces the exchange of GDP with GTP and activates it. The activity of Ras2 is regulated by Ira1 and Ira2, which bind the G protein and stimulate the hydrolysis of GTP to GDP and thus reverse the activation of CDC25. The active complex Ras2/Cdc25 associates and activates, in turn, the adenylate cyclase Cyr1 causing the production of cAMP which binds Bcy1, the regulatory subunit of the cAMP-dependent protein kinase (PKA) (Gagiano et al., 2002). In absence of cAMP (in the absence of glucose), PKA consists in a heterotetrameric complex in inactive state formed by two regulatory subunits and two catalytic subunits. PKA catalytic subunits are three (Tpk1, Tpk2 and Tpk3) and each one displays different functions. Tpk2 is responsible of filamentous growth while Tpk1 and Tpk3 inhibit filamentation (Verstrepen and Klis, 2006; Malcher et al., 2011; Gagiano et al., 2002). Tpk1 exerts its role inhibiting the epistatic Yak1/Sok2/Phd1 pathway that brings to *FLO11* activation (Verstrepen and Klis, 2006; Pan and Heitman, 2000; Malcher et al., 2011) (see below). When cAMP associates with Bcy1, the latter undergo to a conformational change which reduces its affinity for its catalytic subunits and causes their release (Gagiano et al., 2002). Free Tpk2 subunit associates with Sfl1, a transcriptional repressor of *FLO11*, and

phosphorylates it preventing the binding of Sfl1 with the promoter of *FLO11*. On the other hand, Tpk2 exerts its role of activator of filamentous growth activating the transcriptional factor Flo8, which binds *FLO11* promoter together with Mss11 inducing its transcription (Verstrepen and Klis, 2006). Recently, Ryan et al. (2012) identified Mfg1, another regulator of *FLO11* expression which interacts with Flo8 and Mss11 in the promoter of *FLO11*. The other branch of the pathway which brings to cAMP production and PKA activation consists of a G-protein-coupled receptor (GPCR), Gpr1, and a G protein. To date only α and γ subunits have been identified: Gap2 (Gα) and Gpg1 (Gγ). Gpr1 senses sucrose outside the cell and transmits the signal to the G protein activating it and stimulating cAMP production by Cyr1 (Cullen and Sprague, 2012).

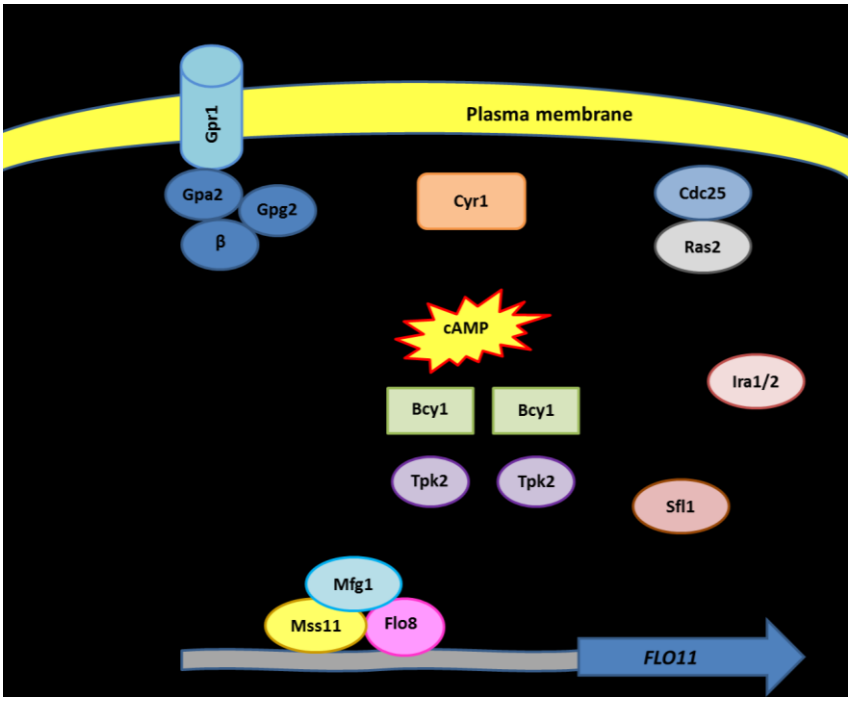


Figure 4: cAMP/PKA pathway. Image adapted from Verstrepen and Klis (2006).

1.8.3 Snf1 glucose-sensing pathway

Another pathway regulates *FLO11* transcription in response to glucose depletion independently from Gpr1/Gpa2/cAMP/PKA pathway: the Snf1 glucose-sensing pathway (Fig. 5). The AMP-activated protein kinase Snf1 formed a complex which consists in an α subunit (Snf1), a β subunit (comprising one of the three isoforms Gal83, Sip1 and Sip2) and a γ subunit (Snf4). During glucose starvation the activating subunit Snf4 binds the

regulatory domain of Snf1 inducing the phosphorylation of its activation loop by an upstream kinase (Busti et al., 2010). Snf1 is activated and phosphorylates the transcription factor Mig1 inhibiting *FLO11* repression (Verstrepen and Klis, 2006). Instead, under high concentrations of sugar, glucose is transported inside the cell by Hxt transporters and phosphorylated by hexokinases to glucose 6-phosphates (Verstrepen and Klis, 2006). The hexokinase Hxk2 promotes the dephosphorylation of Snf1 carried out by the phosphatase complex Glc7/Reg1 bringing to Snf1 inactivation and enabling the transcriptional factors Mig1 and Nrg1/2 to bind *FLO11* promoter (Verstrepen and Klis, 2006; Busti et al., 2010). The formation of the complex Mig1/Nrg1/Nrg2/Tup1/Ssn6 on *FLO11* promoting region represses *FLO11* transcription (Verstrepen and Klis, 2006). Mig1 and its homolog Mig2 regulate also the FG MAPK pathway through their interaction with Opy2. In particular, the glucose-sensing pathway components Mig1, Mig2 and Snf1 activate the FG pathway during glucose starvation while repress it when glucose concentration is higher (Karunanithi and Cullen, 2012). The transcriptional activator Mss11 seems to be required for the derepression of *FLO11* by Nrg1 and Nrg2 (van Dyk et al., 2005).

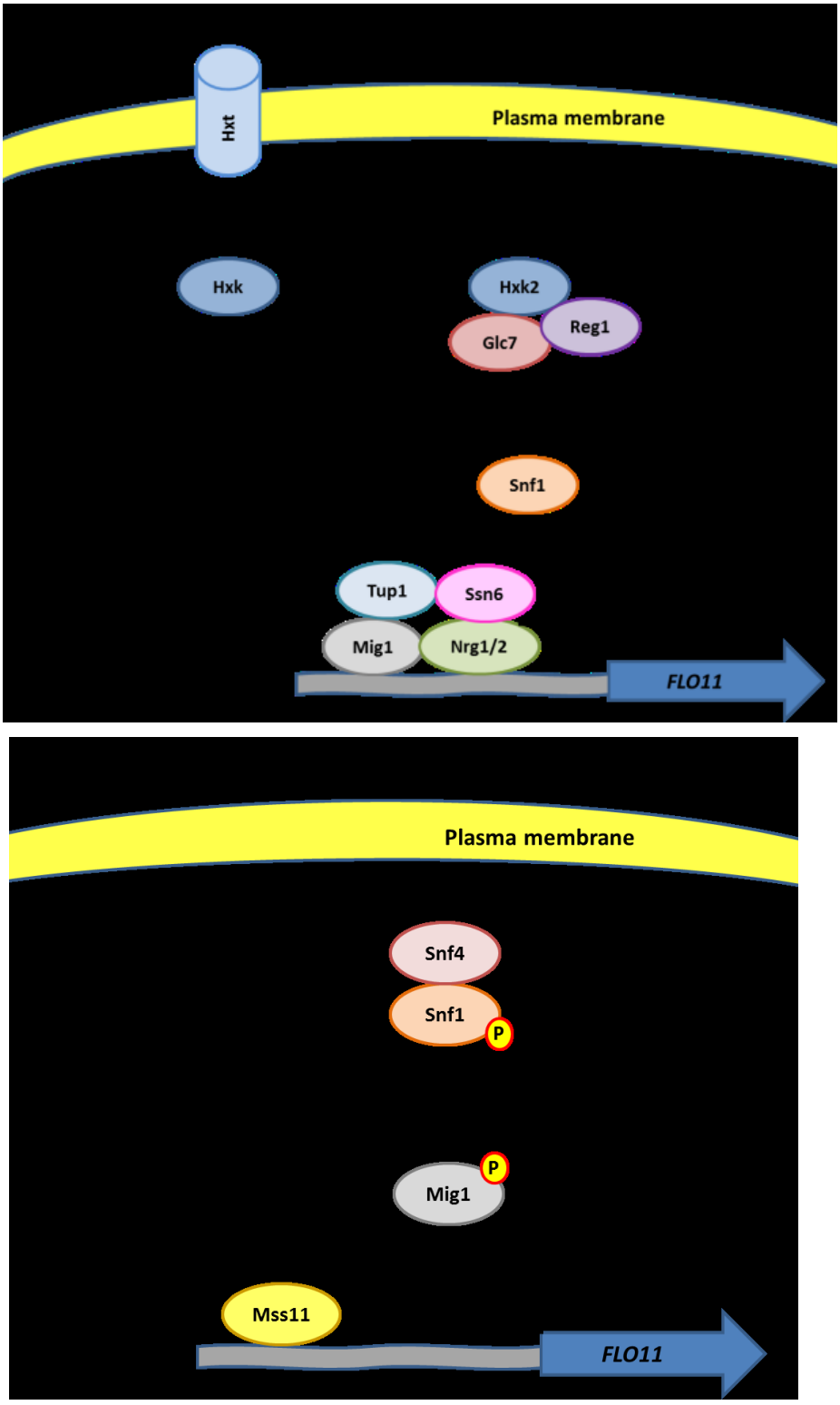


Figure 5: Snf1 glucose-sensing pathway. Image adapted from Verstrepen and Klis (2006).

1.8.4 TOR pathway

The "target of rapamycin" (TOR) pathway has been described to be involved in *FLO11* regulation. The ammonium permease Mep2 captures nitrogen signals (Gagiano et al., 1999)

and transmits them along the TOR pathway regulating *FLO11* expression through the transcription factor Gcn4 (Cullen and Sprague, 2012). Details of this regulation pathway are not yet been understood.

1.8.5 Epistatic pathway

An epistatic pathway has been suggested and involves the transcription factors Sok2, Phd1 and Ash1 (Verstrepen and Klis, 2006). This pathway can be inhibited by the PKA catalytic subunit Tpk1. In particular, when PKA is active Tpk2 activates *FLO11* and Tpk1 inhibits Yak1, very likely phosphorylating it. Phosphorylated Yak1 negatively regulates Sok2, which in turn represses *FLO11* (Malcher et al., 2011) and Phd1, transcriptional activator which together with Mss11 activate the transcription of *FLO11* (van Dyk et al., 2005) . Instead, when PKA complex is inactive Yak1 unphosphorylated can activate the pathway and positively regulate *FLO11*. Furthermore, Yak1 controls *FLO11* regulation also through the transcription factor Haa1 (Malcher et al., 2011) while Sok2 negatively regulates *FLO11* not only directly or through Phd1 but also through the transcription factor Ash1 (Pan and Heitman, 2000).

1.8.6 pH sensing pathway

A pH-sensing pathway has been suggested as further mechanism for *FLO11* activation. This regulation involved the transcription factor Rim101 which is activated by Rim20 (Barrales et al., 2008).

1.8.7 ncRNAs switch of FLO11 expression and epigenetic control

A very specific regulation mechanism triggering *FLO11* expression involves two long intergenic noncoding RNAs, *PWR1* and *ICR1*. When Flo8 binds *FLO11* promoter *PWR1* is transcribed in the opposite direction of *FLO11*, blocking the transcription of the second ncRNA *ICR1*. Instead, *ICR1* transcription occurs in the same direction of *FLO11* when Sfl1 binds *FLO11* promoter. *ICR1* acts covering most of *FLO11* promoting region and inhibiting its expression (Bumgarner et al., 2009). This complex interplay between *ICR1* and *PWR1* has been described as a toggle mechanism. However, the study of the expression of these genes at a single cell level has revealed instead that *ICR1* transcription inhibits recruitment of Flo8 and Sfl1 to the *FLO11* promoter (Bumgarner et al., 2012). Interestingly, this mechanism leads as well to a variegated cell population for the expression of *FLO11*. Epigenetic switch that toggles independently and slowly enable a

large combination of expression states and generate a large phenotypic diversity (Octavio et al., 2009). Silencing of *FLO11* occurs in a SIR independent manner (Halme et al., 2004), and rely on histone deacetylase Hda1. Hda1 may be recruited by Sfl1 to silence *FLO11* in a positional and promoter-specific way (Halme et al., 2004). In addition, the Rpd3L deacetylase and Swi/Snf complexes, respectively responsible of histone modification and chromatin remodeling, affect also promoter chromatin structure and activate *FLO11* transcription. It has been proposed that both complexes act repressing the *FLO11* transcription repressor *SFL1* (Barrales et al., 2008; Barrales et al., 2012). Indeed Rpd3L may act through the repression of its ncRNA *ICR1* (Bumgarner et al., 2009).

Octavio et al. (2009) proposed a global model kinetic explaining variegated *FLO11* expression in cell population resulting from the combination of conventional activation and silencing. They hypothesized a three-status model for *FLO11* expression: silenced (no expression), inactive but competent (no or weak expression) and active state, and different speed for changing status. The switch from silenced to competent is slow, whereas the switch from competent to active (the conventional regulation) is fast. They categorized transcription factors in three categories. Class I contains Tec1, Ste12, and Phd1, which stabilize weakly the competent state with low expression, and the binding sites of these activators are located in nucleosomally occluded regions. The second class of activators, which stabilize the competent state, contains Msn1 and Mss11. Flo8 and Sfl1 represent the third class of activators with opposed action. Flo8, acts in a mode somehow similar to Class II activators. Sfl1 acts as a conventional repressor of *FLO11* and as well as a silencer via Hda1 (Fig. 6).

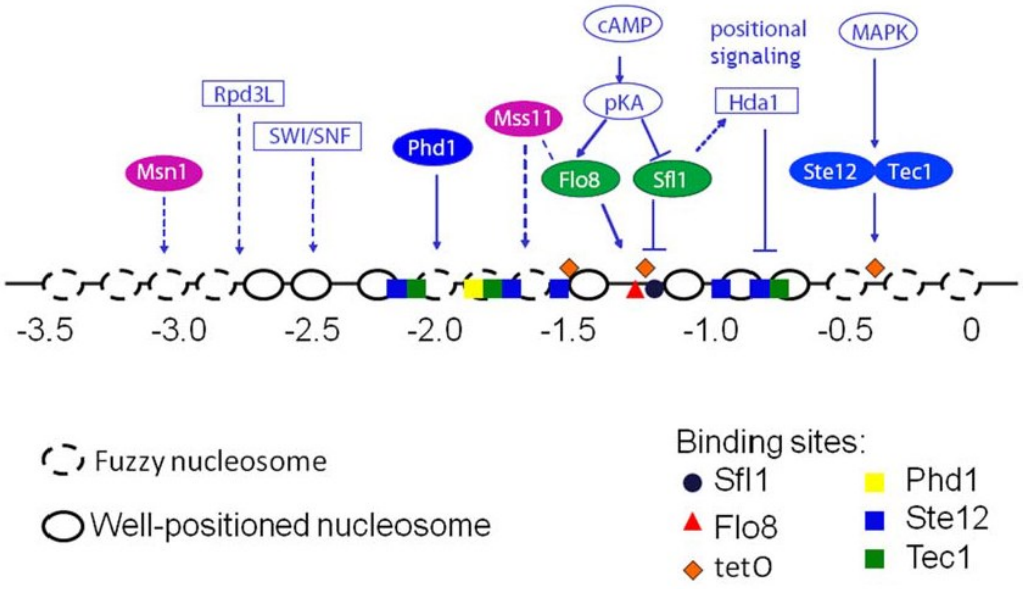


Figure 6: Schematic representation of regulators that converge to *FLO11* promoter. Image from Octavio et al. (2009).

1. CONSTRUCTION OF TOOLS: STRAINS AND SYNTHETIC FLOR MEDIA

The validation of specific genes obtained from a genetic study such as genome sequencing or QTL analysis requires representative strains easy to manipulate genetically, and a growth medium mimicking velum growth on wine to simulate the conditions of flor wines ageing. As some of these tools were not available at the beginning of my PhD thesis, we decided to expand the existing toolbox.

Microsatellite genotyping (Legras et al., 2007) as well RAD-tag sequencing (Cromie et al., 2013) have shown that most laboratory strains (S288c, By4741and By4742, W303, FL100 and FL200, Sigma1278, CENPK113.7D) share the same origin except SK1 and all these strains are not wine strains (http://wiki.yeastgenome.org/index.php/Strains). Along the years, different genetics studies have revealed that these lab strains share deficient alleles for different genes such as *FLO8*, leading to flocculation defects (Kobayashi et al., 1996), *HAP1* leading to have respiratory defects (Gaisne et al., 1999) or *ABZ1* (Ambroset et al., 2011) leading to poor fermentation performances. Sigma1278, a strain widely used for the study of filamentous growth, has a functional version of the *FLO8* gene, but is still not a wine strain. RM11.1a, another haploid strains used in many genetics studies (Brem et al., 2002; Ehrenreich et al., 2010; Ronald & Akey, 2007), has been isolated from a Californian vineyard, but is also a strain with poor ability to ferment (Camarasa et al., 2011). An alternative could have been the use of strains that have been used classically in SPO lab (INRA Montpellier, France) such as V5 (Michnick et al., 1997), a meiotic spore of the industrial strain CIVC8130 (which is a variant of the popular commercial wine strain EC1118). V5 is a good fermenting strain but produces abnormal quantities of some metabolites of central carbon metabolism (Camarasa et al., 2011). More recently 59A, a spore of EC1118 has been chosen for validating the effect of allelic variation at different QTL detected in a cross between EC1118 and S288C (Ambroset et al., 2011). However these two strains, V5 and 59A, present one major disadvantage: as they are derivatives from EC1118 which results from a cross between a wine strain and a flor strain (see chapter 3), their properties cannot be connected to wine or flor genetic background.

For flor strain, the diploid strain 2D has been isolated from a spore of a Sardinian flor strain, and requires to be haploidized in order to simplify the replacement of different genes. However, it must be pointed out that this Mediterranean strain possess several genes which are recombined or deleted in comparison to Jura strains (such as *FRE2-3*) and this strain has a mutated version of *ICR1* non coding RNA that Jura strains P3-D5 do not have

(see chapters 3 and 4). 2D is also an excellent velum grower, and produces a thick velum whereas P3-D5 produces a thin velum that is appreciated by Jura wine makers.

As a consequence we chose to develop a set of haploid strains from the strain that we have sequenced in order to obtain pure wine or flor laboratory strains that will be representative of the different types of strains that we are studying.

In a similar manner, the popular synthetic YNB medium is very easy to handle, but presents high quantities of vitamins that make it too distant from wine composition. As consequence we decided to develop a synthetic wine medium for velum growth to mimic flor wine ageing.

2.1 Materials and methods

2.1.1 Strains

Strains used and obtained in this work are reported in table 1. One strain was obtained by Zara et al. (2010), 4 strains were obtained from INRA-UMR1083 SPO collection, 2 strains were obtained from Spain and 2 from Hungary, 16 strains were obtained in this work.

Table 1: List of strains used and obtained

Strain	Group	Genotype	Origin
F25	Flor		Spain, Cordoba, Prof Mauricio
TA12-2	Flor	MAT**a**/MATα; HO/HO	Hungary, Budapest, Prof Maraz
TS12	Flor	MAT**a**/MATα; HO/HO	Hungary, Prof Maraz
FINO 7.7	Flor		Spain, Prof Cantoral's University of Cadiz
2D	Flor	MAT**a**/MATα; HO/HO; HMRa/HMRa; HMLa/HMLa	(G. Zara et al., 2008)
2D MATa	Flor	MAT**a**; YDL227c::kanMX4	This work
2D MATα	Flor	MATα; YDL227c::kanMX4	This work
P3-D5	Flor	MAT**a**/MATα; HO/HO	This work
P3-D5 MATa	Flor	MAT**a**; YDL227c::kanMX4	This work
P3-D5 MATα	Flor	MATα;YDL227c::kanMX4	This work
P3-D5 MATa	Flor	MAT**a**; ho::loxP	This work
P3-D5 MATα	Flor	MATα; ho::loxP	This work
GUF54-A1	Flor	MAT**a**/MATα; HO/HO	This work
GUF54-A1 MATa	Flor	MAT**a**; YDL227c::kanMX4	This work
GUF54-A1 MATα	Flor	MATα;YDL227c::kanMX4	This work

GUF54-A1 MATa	Flor	MAT**a**; ho::loxP	This work
GUF54-A1 MATα	Flor	MATα; ho::loxP	This work
K1 MATa	Wine	MAT**a**;YDL227c::kanMX4	UMR SPO
K1 MATα	Wine	MATα;YDL227c::kanMX4	UMR SPO
K1 MATa	Wine	MAT**a**; ho::loxP	This work
K1 MATα	Wine	MATα; ho::loxP	This work
D47 MATa	Wine	MAT**a**;YDL227c::kanMX4	UMR SPO
D47 MATα	Wine	MATα;YDL227c::kanMX4	UMR SPO
D47 MATa	Wine	MAT**a**; ho::loxP	This work
D47 MATα	Wine	MATα; ho::loxP	This work

2.1.2 Media and growth conditions

Strains were precultivated in YEPD (2% glucose, 1% yeast extract, 2% peptone) and, after 24 hours, inoculated (OD_{600} = 0.1) in an adaptation medium consisting of YEPD + 3% ethanol (v/v). After 16 hours yeast cells were centrifugated, washed twice to remove any residual sugar from YEPD, resuspended in synthetic flor medium (see table 2) and inoculated (OD_{600} = 0.1) in 50 ml flor medium in 250 ml Erlenmeyer flasks with foam cap. Yeasts were cultivated for 300 hours at 28°C and with 180 rpm agitation. To induce biofilm formation flor strains were precultivated in YEPD overnight, washed twice and resuspended in synthetic flor medium. Three replicates of 2 ml (10^7 cells/ml) of each cellular suspension were transferred in a 24-wells polystyrene plates (flat bottom, 353047, Becton-Dickinson Labware) and incubated in static conditions at 25°C for 4 days.

Table 2: Synthetic flor medium composition. Modified from refermentation medium (Maisonnave et al., 2013):

Compound	Amount in 1000 ml	Compound	Amount in 1000 ml
Glycerol	7 g	Vitamins:	
Ethanol 96%	4% (v/v)	Myoinositol	220 mg
Malic acid	10 g	Calcium pantothenate	0.14 µg
NaOH solution	to pH 3.4	Thyamine hydro	2.2 µg
		Nicotinic acid	0.44 µg
		Pyridoxine	0.12 µg
Minerals:		Biotin	0.6 µg
KH_2PO_4	100 mg	Riboflavin	0.47 µg
K_2SO_4	400 mg		
$MgSO_4 \cdot 7H2O$	300 mg	Amino Acids:	
$CaCl_2 \cdot 2H2O$	35 mg	Proline	800 mg
NaCl	25 mg	Alanine	32.7 mg
$FeCl_3$	5 mg	Arginine	29.8 mg
$ZnSO_4 \cdot 7H2O$	1 mg	GABA	26.2 mg
$CuSO_4 \cdot 5H2O$	0.3 mg	Glutamic acid	24.3 mg
$MnSO_4 \cdot 4H2O$	2 mg	Lysine	16.4 mg
		Leucine	15.7 mg
		Glycine	13.1 mg

		Asparagine Histidine	11.4 mg 7.6 mg

Media used to induce sporulation in strains deleted for *HO* gene were: presporulation medium (1% yeast extract, 0.5% peptone, 10% glucose) and sporulation medium (0.1% yeast extract, 0.05% glucose, 1% potassium acetate, 0.002% adenine).

2.1.3 Creation of haploid strains

Primers used to construct deletion cassette and to verify the deletion are reported in table 3. A 50 µl PCR reaction mix contained: 10 µl 5X KAPA GC Buffer, 0.3 mM KAPA dNTP Mix, 0.3 µM each primer (table 3), 25 ng pUG6 template DNA, 0.5 U KAPA HiFi HotStart DNA Polymerase (KAPAbiosystems). PCR conditions were: 95°C for 3 min, 98°C for 20 sec 60-75°C for 15 sec 72°C for 30 sec/Kb (30 cycles), 72°C for 2 min. The PCR product was used to transform the strains P3-D5 and GUF54-A1 using the protocol described by Güldener and colleagues (1996). Confirmation of *HO* deletion was performed by PCR: initial denaturation at 95°C for 3 min; 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec/Kb (39 cycles); final extension at 72°C for 5 min. A 25 µl PCR reaction mix contained: 2.5 µl 10X Taq Buffer KCl, 0.2 mM each dNTP, 0.3 µM of each primer (table 3), 25 mM $MgCl_2$, 1.20 U Taq DNA Polymerase (Fermentas). Positive transformants were then precultivated in 10 ml of presporulation medium (1% yeast extract, 0.5% peptone, 10% glucose) for 7 hours at 28°C and with 450 rpm agitation. Then, 200 µl of cultures were inoculated in 20 ml sporulation medium (0.1% yeast extract, 0.05% glucose, 1% potassium acetate, 0.002% adenine) and incubated for 2 weeks at RT with slight agitation. Asci were digested with a solution 5% of the enzyme Lyticase (from Arthrobacter luteus, crude. Sigma) for 8 min and spores were separated using a micromanipulator (MSM300 Singer Instrument). Spores bringing *HO* gene deletion were selected on YEPD plates containing 200 µg/ml geneticin G418. Mating type was assigned testing the formation of zygote with haploid references.

Wine haploid strains K1 and D47 were already obtained in the lab INRA-UMR1083 Sciences Pour l'Oenologie with the same method. Kanamycin cassette was removed using the Cre-lox P recombination system (Güldener et al., 1996). The protocol of transformation was the same used for gene deletion but for a Cre-lox P experiment 35 ng (12 µl) of the

plasmid pSH65 was used. Selection of transformants was performed on YEPD plates containing 150 µg/ml phleomycin. Excision of KanMX cassette was confirmed by replica-plate on YEPD and YEPD plus geneticin G418 (colonies that lost resistance were not able to grow on geneticin) and PCR. PCR conditions and reaction mix are described above, primers used were HOdelVerifFOR and REV (table 3).

Table 3: List of primers used

Primer	Sequence	Purpose
HOdelFOR	5' ATGCTTTCTGAAAACACGACTATTCTGATGGCT AACGGTGCTTCGTACGCTGCAGGTC 3'	Obtaining of *HO*::KAN cassette from pUG6
HOdelREV	5' TTAGCAGATGCGCGCACCTGCGTTGTTACCACA ACTCTTTAGTGGATCTGATATCACCTA 3'	Obtaining of *HO*::KAN cassette from pUG6
HOdelVerifFOR	5' TGTTGAAGCATGATGAAGCG 3'	Confirming *HO*::KAN insertion and excision cassette
HOdelVerifREV	5' TGAAACAAATCAGTGCCGGT 3'	Confirming *HO*::KAN insertion and excision cassette
KanP1	5' GCTAAATGTACGGGCGAC 3'	Confirming *HO*::KAN insertion cassette
KanP2	5' TCGCCTCGACATCATCTG 3'	Confirming *HO*::KAN insertion cassette

2.2 Results

2.2.1 Construction of haploid strains

We first deleted the *HO* gene of 3 flor strains, 2D, P3-D5 and GUF54-A1, with a loxP-KanMX-loxP disruption cassette, and obtained Δho haploid spores from tetrads dissection. We removed then the Kanamycin cassette with the help of a plasmid carrying the Cre-loxP recombinase under the control of a *GAL1* promoter (Güldener et al. 1996) in order to be able to use the KanMX marker in further experiments. We obtained haploids of each sexual type MATa and MATα from the strains P3-D5 and GUF54-A1 (table 4). We were not successful to remove the Kanamycin cassette from the flor strain 2D, possibly as this strain is not able to metabolize maltose and galactose (Budroni et al., 2005), which are required to induce the Cre-loxP recombinase on that plasmid. Alternative assays on glycerol as carbon source were not successful as well. Comparison of the *GAL4* gene sequence of flor strains revealed that flor strains had a frameshift in the ORF at position 1860 for most strains and 1797 for 2D leading into a premature stop codon in positions 627 and 617 respectively. This may explains the Gal^- character of flor strains (Braulio Esteve-

Zarzoso, Fernández-Espinar, & Querol, 2004), and the differences between flor strains and 2D in the ability to use the plasmid carrying the Cre-loxP recombinase.

For wine strains we used four haploid strains (D47Δho MATa and MATα, K1Δho MATa and MATα) yet obtained in SPO lab and we transformed them to remove the KanMX cassette in a similar manner as for flor strains (table 4).

Flor strains were chosen of different origins and with different characteristics: strain 2D, isolated in Italy, forms a thick velum, and strains P3-D5 and GUF54-A1 isolated in France, which form a thin biofilm and a thick velum like Sardinian and Spanish strains respectively. GUF54-A1 and 2D carry in the promoter of *FLO11* a deletion of 111bp, corresponding to non coding RNA *ICR1*, a sequence which represses *FLO11* gene (Fidalgo et al., 2006), while the strain P3-D5 does not possess this deletion. Furthermore, in the *ITS1* region P3-D5 presents the insertion of a nucleic acid base C (Charpentier et al., 2009) instead of a 24bp deletion typical of Spanish strains (Esteve-Zarzoso et al., 2001) detected in the strains 2D and GUF54-A1. The ability of these strains to sporulate is another feature that differentiate these strains: P3-D5 sporulates very easily and produces more than 90% viable spores (and for this reason was easier to obtain haploid spores) while 2D and GUF54-A1 sporulate poorly and have a low spore viability according to what described for Sardinian and Spanish strains (Ibeas and Jiménez, 1996; Budroni et al., 2000). P3-D5 carries also an ectopic translocation between chromosomes VIII and XVI which is not shared by other flor strains.

Table 4: List of haploid strains obtained

Group	Original strain	Haploid strain obtained
Wine	K1;MAT**a**;YDL227c::kanMX4	K1MAT**a** Δho
Wine	K1;MATα;YDL227c::kanMX4	K1MATα Δho
Wine	D47;MAT**a**;YDL227c::kanMX4	D47MAT**a** Δho
Wine	D47;MATα;YDL227c::kanMX4	D47MATα Δho
Flor	2D	2D; MAT**a**; YDL227c::kanMX4
		2D; MATα; YDL227c::kanMX4
Flor	P3-D5	P3-D5MAT**a** Δho
		P3-D5MATα Δho
Flor	GUF54-A1	GUF54-A1MATa Δho
		GUF54-A1MATα Δho

2.2.2 Selection of a synthetic medium

Media usually used in the literature, based on YNB/ethanol, are far from wine characteristics, as they present an excess of nitrogen and vitamins. For this purpose we tried to develop a medium which composition was similar to the wine at the end of fermentation, adapting to our needs a synthetic medium recently designed for the study of restart of stuck fermentations (Maisonnave et al., 2013). At the end of wine fermentation glucose and fructose are completely metabolized and ethanol is produced in concentrations up to 16%. Another important by-product of wine fermentation is glycerol (6-10 g/L), which plays a key role for the reoxidation of the NADH excess produced during biomass formation (van Dijken and Scheffers, 1986). During wine fermentation N sources are rapidly consumed except proline, because proline oxidase requires molecular oxygen (Duteurtre et al., 1971; Lasko and Brandriss, 1981; Horák and Ríhová, 1982; Mantachian, 1984). Instead, proline is consumed during biological ageing (Botella et al., 1990; Mauricio and Ortega, 1997).

In this context, we designed a synthetic flor medium using malic acid for buffering, glycerol and ethanol as C-source, and in which minerals, vitamins and amino acids were in concentration similar to the wine at the end of fermentation. The composition of the synthetic flor medium is reported in table 2.

We tested flor growth on this synthetic flor medium on both biofilm-forming conditions and on shaking cultures during non-biofilm conditions (Fig. 1 and 2). All strains tested were able to grow on synthetic flor medium and were able to form biofilm. We tested flor strains from different origins: Spain (F25 and 7_7), France (P3-D5), Hungary (TA12-2 and TS12) and Italy (2D). After 3 days incubation Spanish and Italian strains formed a thick velum (2D, F25 and 7_7) while Hungarian and French a thin one, which is in agreement with what is observed for Jura ageing. In particular, the Hungarian strain TA12-2 formed a very thin velum hardly visible.

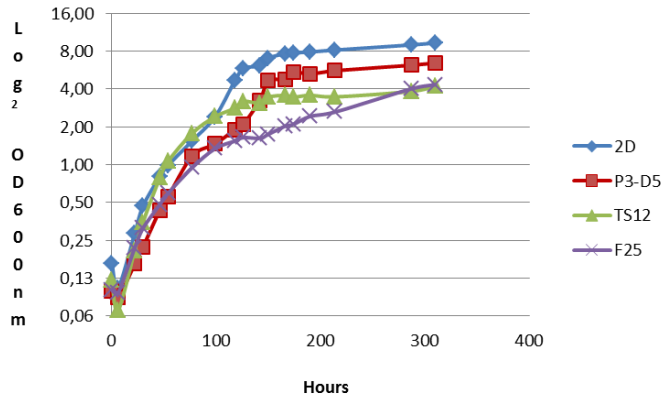


Figure 1: Evaluation of growth on synthetic flor medium under shaking conditions of 4 flor strains.

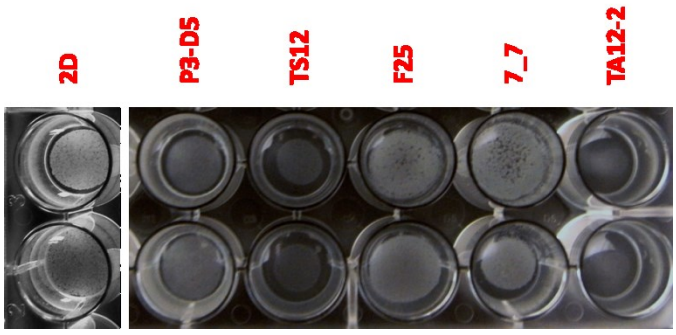


Figure 2: Biofilm formed on synthetic flor medium by 5 flor strains. Experiment was carried out in triplicate.

2.3 Conclusion

We have obtained a set of 3 flor and 2 wine haploid strains for which we have isogenic sexual types that will be available for the study of allelic variations in flor and wine yeasts possibly associated to the differential adaptation to wine fermentation and flor ageing. As the parental strains of these haploid derivatives have been sequenced, they can easily be used for further studies. As an example, the progeny of a cross between a wine and a flor strain could bring valuable complementary information to the results that we obtained through genome sequencing about genes essential for velum build-up. We have also developed a synthetic flor medium which allows growth of flor strains and velum formation, and is as close as possible to wine for velum development studies. This medium will also enable other studies, for example to better explore the differences between thin and thick velum produced by the different strains.

2. IDENTIFICATION AND CHARACTERIZATION OF GENES THAT DIFFERENTIATE FLOR FROM WINE YEASTS

Abstract

Wine fermentation and flor ageing are very different processes carried out by two groups of yeasts able to stand these different lifestyles. One of the most important differences is the ability of flor strains to form a biofilm that allow them to overcome stressing conditions at the end of fermentation. In order to detect specificities involved in the adaptation of flor strains to their ecological niche we sequenced 8 wine strains and 10 flor strains. Population analysis revealed that these groups of strains belong to pure lineages separate one from the other. We detected several divergent regions that may explain the different phenotypes of the strains, and among them four groups of genes were more frequent: genes coding for parietal proteins (*FLO1*, *FLO5*, *FLO9*, *FLO11*, …), metal transporters (*ZRT1*, *ZRT3*, *SMF1*, *ALR1*) and genes implicated in metal homeostasis, genes involved in metabolism (*HXT3*, *ARO10*, *PDC1*), and the fourth group, contained genes participating to the regulation of filamentous growth such as *RGA2*, *STE7*, *IRA1* and *IRA2*. *HXT3* flor allele corresponds to a fructophilic allele of *HXT3* previously characterized. One of the main points of this study is that numerous mutations lead to the deregulation of *FLO11* expression, suggesting that they are the hallmark of flor domestication.

Introduction

The yeast *S. cerevisiae* is one of the most popular eukaryote model, and it is also responsible of a lot of biotechnological processes such as fermentation of wine, beer and bread (Legras et al., 2007). Man's use of this yeast species for the manufacture of fermented products is very ancient: a Chinese fermented beverage has been dated 7000 BC while the earliest remains of wine found in Iran have been dated back to 5400-5000 BC (McGovern et al., 1996; McGovern et al., 2004). From this Mesopotamian based origin, vine culture (Arroyo-Garcia et al., 2006) and wine production and consumption have expanded all over the world. From 2000 BC wine was produced in Greece and Crete but its spread along the Mediterranean, started around 1000 BC, was achieved during Romans colonization, who carried it also in Germany and Northern Europe (Pretorius, 2000). This antique, even if unconscious, link with human activity has led to the debate if *S. cerevisiae* is a species derived from human domestication or if it was present in nature and selected by humans (reviewed by Sicard and Legras, 2011).

Fay and Benavides (2005) supported the hypothesis that *S. cerevisiae* has natural origins followed by domestication. They speculated two domestication events: one, dated 3800 years ago, for saké strains and the second, dated 2700 years ago, responsible of divergence inside wine strains, whereas the divergence between these two population was 11,900 years ago. However, these estimations are based on a continuous growth with a short doubling time of 3 hours. A ten time higher estimation can be postulated, leading to estimates in agreement with those obtained by Legras et al. (2007) who hypothesized a co-migration of wine yeast from Mesopotamia with vine.

The environment created by winemaking techniques impose a selective pressure on wine yeasts which leads to adaptive evolution mechanisms responsible of genetic characteristics of wine strains, stress adaptation, gene expression variability, genomic rearrangements and interspecific hybridization (reviewed in Querol et al., 2003).

Mutation is an important mechanism of adaptive evolution as it generates diversity that may be further captured by selection. For instance, sulphite is a compound toxic for yeast cells, and the use of sulphur fumes dates back at the time of Egyptian, Greek and Roman civilizations for wine containers washing. The addition of SO_2 to the must was further carried on as a preservative since the Middle Ages. These winemaking techniques have led to the selection of a *SSU1* translocation between chromosome VIII and XVI, which increases resistance to sulfite (Pérez-Ortín et al., 2002).

Flor ageing is also a harsh environment to which flor yeasts are specifically adapted, and has been proposed as an example of yeast domestication (Legras et al., 2007). After the end of fermentation these special strains rise at the wine surface forming multicellular aggregates that lead to the build-up of a biofilm, necessary for the biological ageing of white wines like Sherry (Spain), Vin Jaunes (France), Tokaji (Hungary) and Vernaccia (Italy). In aerobic conditions flor yeasts show high resistance to ethanol which becomes the main carbon source during biofilm formation and is oxidized to acetaldehyde and then to acetic acid (Aranda and del Olmo, 2003). Ethanol is a major cause of stress for cell (Stanley et al., 2010), as well as acetaldehyde (Ristow et al., 1995; Castrejón et al., 2002; Aranda et al., 2002), that flor strains are able to overcome thanks to metabolic and genetic peculiarities (Budroni et al., 2005). It has been hypothesized that this adaption should be related to mutations. Indeed, acetaldehyde causes double-strand breaks (DSBs) in DNA (Ristow et al., 1995) which are considered responsible of the mitochondrial DNA

polymorphism in flor yeasts (Castrejon et al., 2002) and of gross chromosomal rearrangements (GCRs) (Infante et al., 2003). Infante et al. (2003) proposed flor strains as a model for speciation studies of *S. cerevisiae*, identifying GCRs as important factor for adaptive evolution of natural strains: genes contained inside these duplicated regions may have a higher expression as a result of gene dose effect that could be useful for flor characteristics. However, recently CGH array results obtained on flor strains from Hungary, Spain, France and Italy, infirm this hypothesis (Legras et al., submitted) as they show few duplicated genes in the genome of 7 flor strains that they compared by CGH on array. Fidalgo et al. (2006) suggested a model of adaptive evolution of flor strain based on mutations in the gene *FLO11*. They associated the acquisition of floatability to the combination of mutations in the promoter and in the coding region: a deletion of 111bp in the promoter which comprises a repression sequence necessary to the inhibition of *FLO11* that leads to a higher expression levels and a minisatellite expansion which amplification corresponds to an increased hydrophobicity.

In this study we sequenced the genome of 18 yeasts (wine and flor) and of the industrial strains EC1118 and 59A in order to detect genes potentially involved in the adaptation of flor strains to biological ageing conditions of Sherry-like wines. We identified several genomic variations in genes coding for cell wall proteins, repressors of filamentous growth or involved in metal homeostasis and metabolism. We assessed the phenotypic impact of some allelic variants.

Materials and methods

Yeast strains, media and growth conditions

Yeast strains used in this work and their origin are listed in table 1a and b.

Table 1a: List of strains used for sequencing

Strain	Origin	Form sequenced	Sequencing depth
GUF54-A1	Jura, France	Spore, 2n	246
P3-D5	Jura, France	Spore, 2n	107
TA12-2	Tokaji, Hungary	Spore, 2n	198
TS12-A7	Tokaji, Hungary	Diploid wild-type	292
2D	Sardinia, Italy	Spore, 2n	430
7-7	Jerez, Spain	Diploid wild-type	346
F25	Cordoba, Spain	Triploid wild-type	731
F12	Cordoba, Spain	Diploid wild-type	392

VPDN	Jerez, Spain	Diploid wild-type	100
MJ73	Lebanon	Diploid wild-type	100
K1_ 28-1A spore from K1 (MTF 1832)	Languedoc, France	Spore, 2n	252
D47_6-4A spore from D47 (MTF 1833)	Languedoc, France	Spore, 2n	143
1014-F5 (MTF 2119)	Tuscany, Italy	Spore, 2n	124
GE7-4A (MTF 2281)	Alsace, France	Spore, 2n	410
22A4 spore from L2226 (MTF 2396)	Rhone Valley, France	Spore, 2n	230
20B2 spore from L2056 (MTF 2395)	Rhone Valley, France	Spore, 2n	293
L1414 (MTF 2113)	Beaujolais, France	Diploid wild-type	77
6320-A7 (MTF 2295)	Sardinia, Italy	Spore, 2n	285
EC1118	Champagne, France	Diploid wild type	100
59A spore of EC1118	Champagne, France	Spore, 2n	100

Table 1b: List of derived strains used for the evaluation of allelic variation

Strain	Group	Genotype	Origin
P3-D5	Flor	MATα ; ho::loxP	(Coi et al., chapter 2)
K1	Wine	MATa ; ho::loxP	(Coi et al., chapter 2)
K1 Δzrt1	Wine	MATa ; ho ::loxP ; YGL255W::loxP-kanMX-loxP	This work
P3-D5 Δzrt1	Flor	MATα ; ho ::loxP ; YGL255W::loxP-kanMX-loxP	This work
P3-D5 *ZRT1*-K1	Flor	MATα ; ho ::loxP ; YGL255W:: YGL255W (K1)	This work
K1 *ZRT1*-P3-D5	Wine	MATa ; ho ::loxP ; YGL255W:: YGL255W (P3-D5)	This work

The media used to evaluate *ZRT1* phenotypes were YEPD, YEPD agar + 6 mM H_2O_2, YEPD agar + 2 mM H_2O_2 + 1 mM EDTA, YNB w/o amino acids + 1mM EDTA pH 4.2 + 2% agar (Zhao and Eide, 1997), synthetic flor medium (table 2) 8% ethanol without $ZnSO_4$ and 10 times more vitamins, YNB + 4% ethanol + EDTA or H_2O_2.

For genomic microarray experiments yeasts were precultivated in YEPD (2% glucose, 1% yeast extract, 2% peptone) and, after 24 hours, inoculated (OD_{600} = 0.1) in YEPD + 3% ethanol (v/v). After 16 hours strains were centrifugated, washed twice, resuspended in synthetic flor medium (see table 2) and inoculated (OD_{600} = 0.1) in 50 ml flor medium in 250 ml Erlenmeyer flasks with foam cap. Yeasts were cultivated to reach an OD=0.8 at 28°C and with 180 rpm agitation.

Table 2: Synthetic flor medium composition. Modified from refermentation media (Maisonnave et al., 2013):

Compound	Amount in 1000 ml	Compound	Amount in 1000 ml
Glycerol	7 g	<u>Vitamins:</u>	
Ethanol 96%	4% (v/v)	Myoinositol	220 mg
Malic acid	10 g	Calcium pantothenate	0.14 µg
NaOH solution	to pH 3.4	Thyamine hydro	2.2 µg
		Nicotinic acid	0.44 µg
		Pyridoxine	0.12 µg
<u>Minerals:</u>		Biotin	0.6 µg
KH_2PO_4	100 mg	Riboflavin	0.47 µg

K_2SO_4	400 mg		
$MgSO_4 \cdot 7H2O$	300 mg	Amino Acids:	
$CaCl_2 \cdot 2H2O$	35 mg	Proline	800 mg
NaCl	25 mg	Alanine	32.7 mg
$FeCl_3$	5 mg	Arginine	29.8 mg
$ZnSO_4 \cdot 7H2O$	1 mg	GABA	26.2 mg
$CuSO_4 \cdot 5H2O$	0.3 mg	Glutamic acid	24.3 mg
$MnSO_4 \cdot 4H2O$	2 mg	Lysine	16.4 mg
		Leucine	15.7 mg
		Glycine	13.1 mg
		Asparagine	11.4 mg
		Histidine	7.6 mg

Genome sequencing, population analysis and SNP's detection

Genomic DNA samples from 10 flor strains, eight wine strains, EC1118 and 59A were prepared following a classical phenol chloroform extraction protocole, separated from mitochondrial DNA after isopycnic CsCl ultracentrifugation (centrifugation 289000g during 20h) (Barth & Gaillardin, 1996) and processed to generate libraries of short 300-bp inserts. Having passed quality control, the 20 libraries were multiplexed in different lanes of an Illumina HiSeq 2000 platform. Sequencing from both ends generated paired-end reads of 2x100 bp, resulting in an average sequencing depth between 77 and 730X. Image analysis and data extraction were performed using Illumina RTA version 1.13.48.0 and CASAVA version 1.8.2. Raw sequence fastq files will been deposited in the Sequence Read Archive (SRA) database. Reads sequences were processed in order to improve their gobal quality: they were trimmed for the first 9 nucleotides using FASTX Toolkit version 0.0.13 and for low quality regions using Sickle version 1.000 (quality below 25 in a 20bp sliding window). Reads shorter than 50 bp were further removed.

Filtered short reads were processed with SOAP*denovo* v1.05 (Li et al., 2010) using different Kmers, and the assembly with the best N50 was selected for each strain. These assemblies were aligned pairwised to S288C reference genome sequence and to all other genome sequences available from SGD http://www.yeastgenome.org/ (Engel and Cherry, 2013), using MUMmer 3.0 (Kurtz et al., 2004).

We used the Genome Analysis Toolkit (GATK) (McKenna et al., 2010) version 2.3-9 for making SNP and indel calls, following "Best Practice Variant Detection with GATK v4, for release 2.0" available online. Briefly, the workflow is divided into four sequential steps: initial mapping, refinement of the initial reads, multi-sample indel and SNP calling, and

finally variant quality score recalibration. First, reads were aligned to the S288c reference genome (release number R64-1-1, downloaded from SGD) using BWA version 0.6.2 (Li & Durbin, 2009) resulting in aligned reads in a BAM file format. Second, optical and PCR duplicates were removed using MarkDuplicate from the Picard Tools version 1.84 (http://picard.sourceforge.net). Reads around indel were realigned to increase indel call accuracy using IndelRealigner (GATK). Base quality were recalibrated using BaseRecalibrator/PrintReads (GATK). After recalibration, the recalibrated base quality scores in the output BAM file are closer to their actual probability of mismatching the reference genome, and are subsequently more accurate. Moreover, the recalibration tool attempts to correct for variation in quality with machine cycle and sequence context. At the end of this step we get the analysis-ready reads. Third, we performed SNP and indel discovery across all evolved and ancestral sequences simultaneously using UnifiedGenotyper (GATK). Fourth, we used Variant Quality Score Recalibration (VQSR) to build an adaptive error model using known variant sites and then applied this model to estimate the probability that each variant in the callset is a true genetic variant or a machine/alignment artifact. This step was performed with the VariantRecalibrator/ApplyRecalibration tools of GATK, using an unpublished dataset of known SNP and indel obtained from 86 available genomes to train the model. This genotyping pipeline provided us a final multi-sample VCF file containing all variant sites discovered across samples to which were associated a genotyping quality for each strain. This set of variant were then further phased with Beagle 3.3 (Browning and Browning, 2007). For compatibility of data format with downstream programs, the vcf files were converted to plink format using plink v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al., 2007).

Phylogeny was obtained from the count of variant single nucleotide polymorphic positions (SNP) found in the alignments between each pair of strain. Phylogenic trees were obtained with Mega 5.0 (Tamura et al., 2011), and single genes sequences were aligned with the built in clusterW module included in MEGA. Structure analyzes were performed applying the R package adegenet (Jombart and Ahmed, 2011; Jombart et al., 2010) of the R v3.0.2 software (R Development Core Team, 2011) and admixture (Alexander et al., 2009), to the set of variant positions obtained from the assemblies. Other analyzes were performed from the VCF variant file: PCA was computed with adegenet, and Nucleotide diversity and

Tajima's D were computed with vcftools (v_0.1.11) (Danecek et al., 2011). McDonaldKreitman standard and generalized tests were performed at http://mkt.uab.es (Egea et al., 2008).

Construction of mutants

Primers to construct deletion cassette and to verify the deletion are reported in table 3. A 50 µl PCR reaction mix contained: 10 µl 5X KAPA GC Buffer, 0.3 mM KAPA dNTP Mix, 0.3 µM each primer (table 3), 25 ng pUG6 template DNA, 0.5 U KAPA HiFi HotStart DNA Polymerase (KAPAbiosystems). PCR conditions were: 95°C for 3 min, 98°C for 20 sec 60-75°C for 15 sec 72°C for 30 sec/Kb (30 cycles), 72°C for 2 min. The PCR product was used to transform the strains P3-D5 and K1 using the protocol described by Güldener and colleagues (1996). Confirmation of allele deletion was performed by PCR: initial denaturation at 95°C for 3 min; 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec/Kb (39 cycles); final extension at 72°C for 5 min. A 25 µl PCR reaction mix contained: 2.5 µl 10X Taq Buffer KCl, 0.2 mM each dNTP, 0.3 µM of each primer (table 3), 25 mM $MgCl_2$, 1.20 U Taq DNA Polymerase (Fermentas).

Table 3: List of primers used

Primer	Sequence	Purpose
ZRT1KanFw	5' ATGAGCAACGTTACTACGCCGTGGTGGAAACA ATGGGACCCTTCTTTCGTACGCTGCAGGTCGAC 3'	Obtaining *ZRT1*::KAN insertion cassette
ZRT1KanRv	5' TTAAGCCCACTTACCGATCAAAGCCATGATACC GCACCGAAAAGGCATAGGCCACTAGTGGATCT 3'	Obtaining *ZRT1*::KAN insertion cassette
ZRT1verFw	5' GCTCAGGTATCTTCTCTCCAATGA 3'	Confirming *ZRT1*::KAN insertion cassette and amplifying ZRT1 allele
ZRT1verRv	5' TATTCTGGTACCACTTGTGCACAC 3'	Confirming *ZRT1*::KAN insertion cassette and amplifying ZRT1 allele
ZRT1verif2Fw	5' CGTTACCGTCAGTTTTCTTCA 3'	Confirming ZRT1 allelic replacement
ZRT1verif2Rv	5' TCGTAGATGGCACGGTCTTAT 3'	Confirming ZRT1 allelic replacement
KanP1	5' GCTAAATGTACGGGCGAC 3'	Confirming KAN insertion cassette
KanP2	5' TCGCCTCGACATCATCTG 3'	Confirming KAN insertion cassette

Primers used for *ZRT1* mutants were: ZRT1KanFw and ZRT1KanRv for deletion, ZRT1verFw, ZRT1verRv, KanP1 and KanP2 to confirm insertion of Kanamycin cassette. ZRT1verFw and ZRT1verRv were also used to amplify allele for allelic replacement, while ZRT1verif2Fw and ZRT1verif2Rv to verify allelic replacement. This allelic replacement was performed in two steps: deletion of *ZRT1* with a Kanamycin cassette and replacing this with the desired functional allele (table 4). As deleted mutants are unviable during zinc

deficiency, selection of allelic replacement mutants was carried out in a low zinc medium (YNB + EDTA 1mM pH 4.2, Zhao and Eide, 1997).

Table 4: List of *ZRT1* mutants constructed

Group	Original Strain	Strain obtained
Flor	P3-D5	P3-D5Δzrt1
Flor	P3-D5 Δzrt1	P3-D5 *ZRT1*-K1
Wine	K1	K1 Δzrt1
Wine	K1 Δzrt1	K1 *ZRT1*-P3-D5

Evaluation of ZRT1 phenotype

For oxidative stress assay strains were precultivated overnight on YEPD, washed twice with sterile water and 4 µl of a 10^7cells/ml cell suspension were spotted on YEPD, YEPD + 6 mM H_2O_2 and YEPD + 2 mM H_2O_2 + 1 mM EDTA plates. Petri dishes were incubated 3 days at 25°C.

Evaluation of phenotype on solid low zinc media was performed on YNB w/o amino acids + 1mM EDTA pH 4.2 + 2% agar (Zhao and Eide, 1997). Cells were precultivated overnight on YEPD, washed twice with sterile water and 4 µl of 2 different dilutions (10^6 and 10^5 cells/ml) were spotted on the plate. Plates were incubated 3-5 days at 25°C. Growth on liquid media was evaluated on synthetic flor medium (table 2) 8% ethanol without $ZnSO_4$ and 10 times more vitamins. Strains were precultivated on YEPD, after 16 hours, inoculated (OD_{600} = 0.1) in YEPD + 5% ethanol. After 9 hours strains were washed twice, resuspended in synthetic flor medium and inoculated (OD_{600} = 0.1) in 50 ml flor medium without $ZnSO_4$. Yeasts were cultivated for 300 hours at 28°C and with 180 rpm agitation, in 250 ml flasks with foam cap.

Ability to form biofilm was evaluated on YNB + 4% ethanol. Strains were precultivated overnight on YEPD, washed twice and resuspended on YNB + 4% ethanol. Four replicates of 2 ml (10^7 cells/ml) were aliquoted on 24-wells polystyrene plates (flat bottom, 353047, Becton-Dickinson Labware) and incubated in static conditions at 28°C for 3 days. Biofilm-forming ability on zinc limited media and under oxidative stress conditions was evaluated adding 2, 5, 10 mM EDTA and 1, 2, 3 mM H_2O_2 respectively before inoculum.

Microarray analysis

RNA extraction was performed with a version slightly modified of the protocol "TRIZOL® Plus RNA Purification Kit" (cat. no. 12183-555, Invitrogen). Briefly: 100 µl trizol and 100 µl glass beads were added to frozen pellets and vortexed for 10 min at 2500 rpm at 4°C. Then, 900 µl trizol were added and tubes were vortexed again for 5 min at 2500 at 4°C. 200 µl chloroform were added, tubes were vortexed for 15 sec and incubated for 3 min at RT. Samples were centrifuged at 13000 rpm for 15 min at 4°C and centrifuged for 1 additional min at 4500 rpm using a swinging bucket centrifuge. 350 µl of upper phase were transferred in a new tube, 350 µl of ethanol 70% were added and tubes were inverted. Then, samples were purified according to the protocol "TRIZOL® Plus RNA Purification Kit". RNA was spectrophotometrically quantified and the quality was checked with the Agilent 2100 Bioanalyzer. To label cDNA was used the kit "Low input Quick Amp Labelling one-color" (cat. no. 5190-2305, Agilent Technologies). Probes obtained were purified with the kit RNeasy Mini Kit (cat. no. 74104, Qiagen) and their quality was checked with the Agilent 2100 Bioanalyzer. Labels were hybridized to yeast whole genome microarrays (cat. no. G2509F, 8x15K, Agilent Technologies) using the Gene Expression Hybridization Kit (cat. no. 5188-5242, Agilent Technologies). The hybridization signal was detected with a GenePix 4000B laser Scanner (Axon Instruments). Acquisition and quantification of array images were performed using the integrated GenePix software Pro7. Statistical analysis was performed using R.2.15.2 (R Development Core Team, 2008). The limma package (Smyth and Speed, 2003; Smyth, 2004; Smyth et al., 2005) was used to import, normalize and analyze the microarray data. The quantile method was used for the normalization between arrays. One comparison was made: K1 versus P3-D5. To determinate the differential gene expression between experimental conditions, a modified t-test was performed by filtering on confidence at $p < 0.05$, using the Benjamini and Hochberg false discovery rate as multiple testing corrections of the t-test p-values (Benjamini and Hochberg, 1995). The genes with different expression were grouped according to Gene Ontology (GO) process terms using YeastMine (Balakrishnan et al., 2012). Hierarchical clustering was used to group the selected genes by similarity in expression profile using cluster version 3.0 (applying centered correlation and complete-linkage clustering) and results were loaded into JavaTreeView version 1.1.5r2 for data display (Saldanha, 2004; de Hoon et al., 2004).

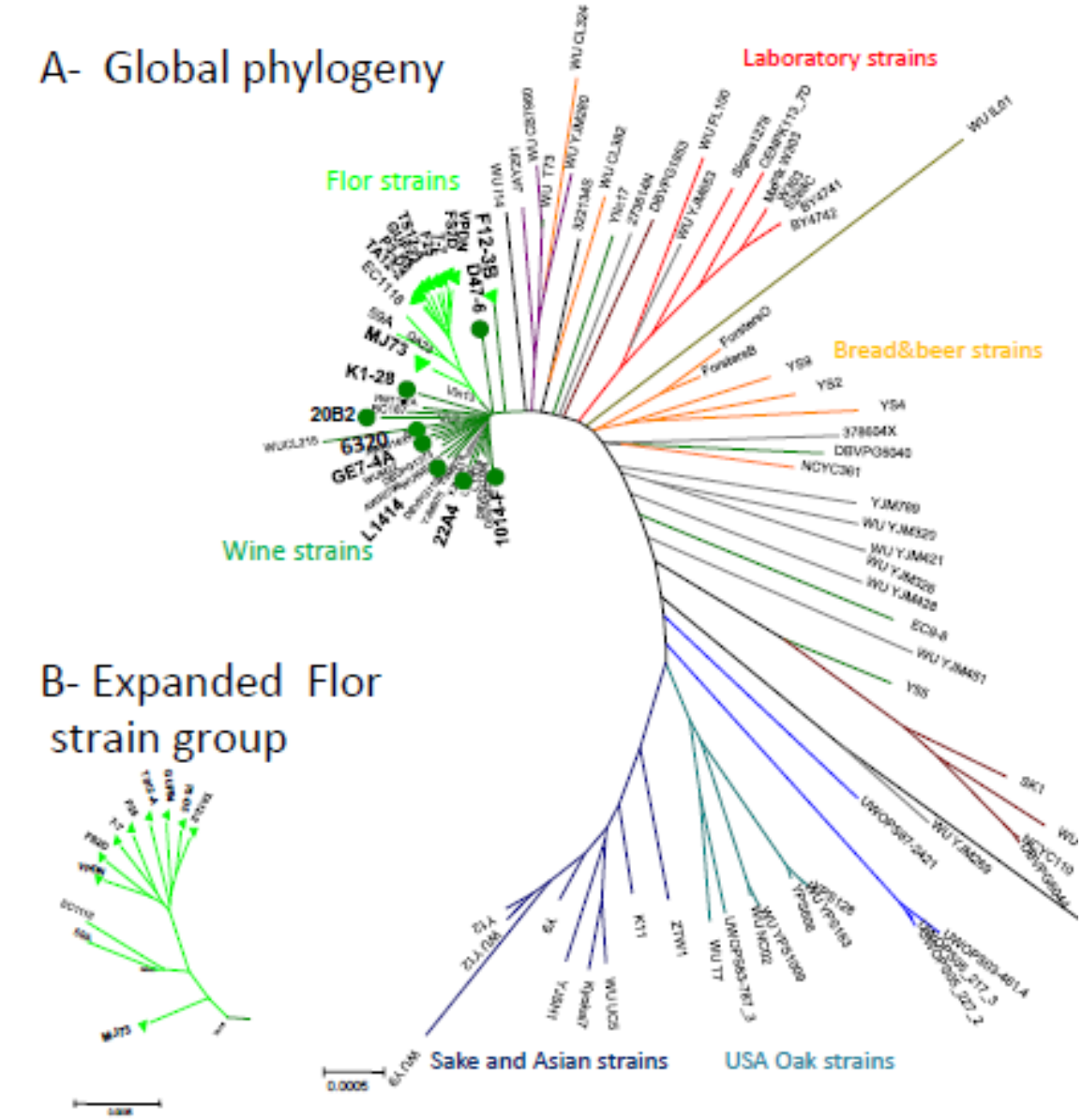
Results

Flor strains phylogeny

As a first step to visualize the phylogenetic relationships between flor, wine and other yeast strains, we aligned to S288c genome sequence the genome sequences of the 10 flor, 8 wine, EC1118 and 59A as well as 84 available genome sequences available from SGD or other databases. We obtained 1033207 variant positions with MUMmer that were reduced to 813666 biallelic positions after removing positions with missing data for more than 5 individuals as well as multiallelic positions. From the table containing only biallelic position, we could build a neigbour joining tree. The global topology of the tree (Fig. 1A) is in good agreement with previously published data (Liti et al., 2009) and includes the different lineages described: wine, sake, oak, Malaysian, African palmwine. Seven out of eight wine sequenced strains were included in the wine cluster, and eight of the ten flor strains clustered very closely, above the wine cluster, besides F12-3B flor strain which behaves atypically. Flor strains have a mean nucleotidic divergence of 0.00175 per bp in the alignment with wine strains, whereas the mean divergence among wine strains or flor strains is 0.00116 per bp and 0.00070 per bp respectively. Interestingly, we could easily differentiate "Central Europe" strains (Hungary and France) from "Mediterrannean" strains (Spain and Sardinia) (Fig. 1B). The industrial strains EC1118, and QA23 appear as related to the group of flor strains, and Lebannon strain MJ73 has a basal position in the cluster containing flor strains. One flor strain, F12-3B, does not belong to the main flor strain cluster nor to the wine, but is still related to these two groups.

As the cluster of flor strains are found close to wine strains, we decided to evaluate if this cluster is individualized and represents a separate lineage from wine yeast or an admixture between different lineages. To evidence population structure, we submitted a subset of 105150 SNPs obtained after removing positions with more than 10 missing data and pruning linked loci, to population structure inference with the admixture software (Alexander et al. 2009), which relies on a similar algorithm as the popular structure software (Pritchard et al., 2000) but performs much faster analysis. An optimum solution of nine groups was inferred (Fig. 1C), that separates Flor strains from Wine and other origins. A similar result was obtained as well with the DPCA R package (Jombart, 2008), which does not make any assumption on *S. cerevisiae* life cycle. Interestingly inside the flor group, EC1118, 59A, QA23, MJ73 and VIN13, appeared as mosaic genomes between flor

and wine strains (bars with two nuances of green) with a growing gradient of “wine yeast genome” content.



C- Inference of population structure

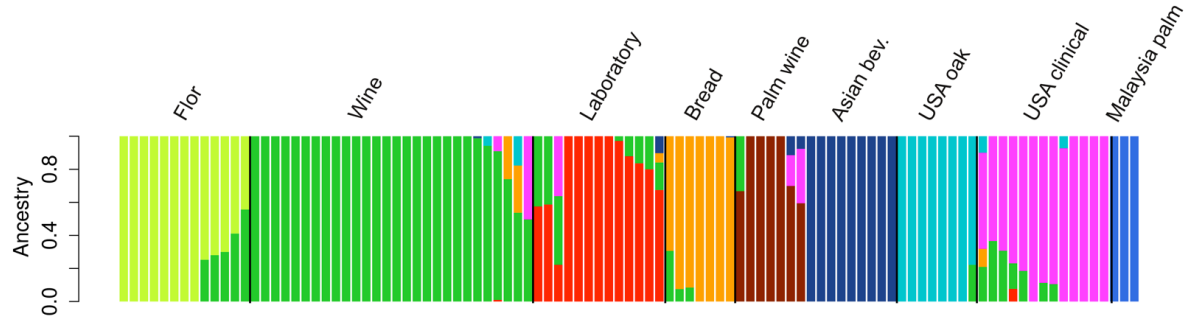


Figure 1 Phylogeny of flor and wine strains. A: Phylogenic tree presenting the position of the 10 flor strains and 8 wine strains sequenced in comparison to other groups of strains. Strains sequenced here are indicated with a circle for wine strains and a triangle for flor strains. Flor strains are colored in bright green, wine strains in green, sake and Asian strains in dark blue, Oak strains in blue-green,

bread and beer strains in orange, laboratory strains in red, African strains in brown. B: Expanded view of the flor strain cluster: TA12 and TS12 (Hungary) and GUF54 and P3-D5 (France) are found in the right branches of the cluster whereas 7.7, VPDN and F25 are found in the left branches of the cluster. C: Clustering of the different strains obtained with Kmeans method (R-adegenet package (Jombart et al., 2009)).

Search for regions differentiating wine and flor strains possibly explaining flor yeast adaptation

The comparison of the genome of 20 strains to S288c reference genomes enabled us to detect 133207 variants (indels, bilallelic and multi allelic positions) withMUMmer, which were further brougth down to 81942 high quality biallelic positions. In order to detect regions possibly involved in the differentiation between wine and flor strains and which may explain differential adaptation to the two ecological niches, we used three different approaches based on the genomewide set of variants: a Principal Component Analysis, a genomescan of Nucleotide diversity π, and of Tajima's D. The diversity of the different strains can be seen as a multidimensional space created by SNPs (as variables), that can be summarized by a Principal Component Analysis (PCA), which computes independent synthetic variables from the set of SNPs. SNPs that have the highest contribution to the axis differentiating the two groups of strains can be then retained (Jombart et al., 2010). In comparison to PCA, Nucleotide diversity enables the detection of regions which have accumulated specifically a high number of mutations specific to each population. The last test Tajima's D relies on the comparison of two measures of nucleotic diversity under a neutral model of evolution: Nucleotide diversity and Watterson's Θ estimate. Under this hypothesis, a negative value of D may indicate a recent positive selection, or a rapidly growing population whereas positive estimates of D may indicate balancing selection or population structure. In our case, the differentiation of the two populations should lead to an inflation of D.

Two Principal Component Analysis (PCA), performed on the whole set of SNPs or on high quality biallelic variants led to similar results. The PCA obtained for high quality biallelic positions (Fig. 2) or from the whole set of variant (unshown data) enabled us to differentiate the two groups of wine and flor strains as well as the atypical flor strain F12. EC1118, its haploid derivative 59A, and the Libanese strain MJ73 have a median position between wine and flor in agreement with the hypothesis that they result from a cross between a wine and a flor strain. As too many SNPs contribute to the differentiation of wine and flor strains, and no clear outliers can be detected, we used instead the summed

contribution of SNPs contained by 500bp regions, in order to detect regions capturing most of the differences. We selected 1% of the regions presenting the highest contribution to PCA axis, and ended up with two set of 146 and 136 genes from to the whole set of variants or from the set of high quality biallelic variants respectively. In this second set, as SNPs are filtered on their quality score, several positions are removed from the whole set of variants which leads to noticeable differences in the two gene lists.

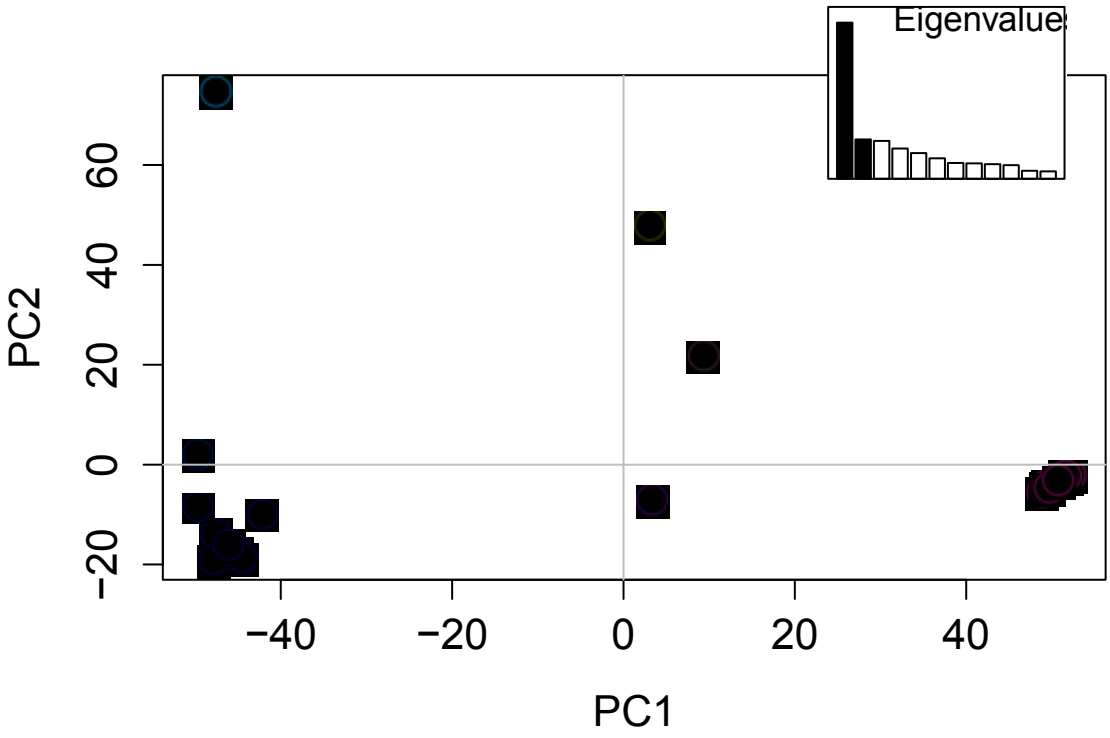


Figure 2: Principal component analysis built on the different variants. The 1st and 2nd axis (PC1, PC2) represent 37.6% and 9.5% of the gobal variance respectively. Strains are colored according to their contribution to the axis.

When comparing these two set of genes, it is noteworthy to see that the 91 genes specific to the whole set of variants contain 6 retrotransposon TYA Gag and TYB Pol genes, genes of unknown function, and genes coding for parietal proteins such as *FLO1*, *5*, *11*, *PAU4*, *6*, *8*, *11*, or *HKR1*. Such repeated genes or genes that have been reported to contain internal repeats (Verstrepen, Jansen, Lewitter, & Fink, 2005) lead to poor read alignments on reference genome, and therefore poor quality mapping which explain the lower score of the

variants in the whole set. However, a few genes that contain highly divergent regions do not belong to that class: *HXT3*, *FRE3* or *PDC1*, and deserve a specific interest.

Interestingly it is noteworthy that the regions differentiating the two populations are localized in some divergent regions and several of them are located in subtelomeric regions such as the end of chromosome I, V, VII and IX (Fig. 3A and B). Both lists of genes are given in table 6.

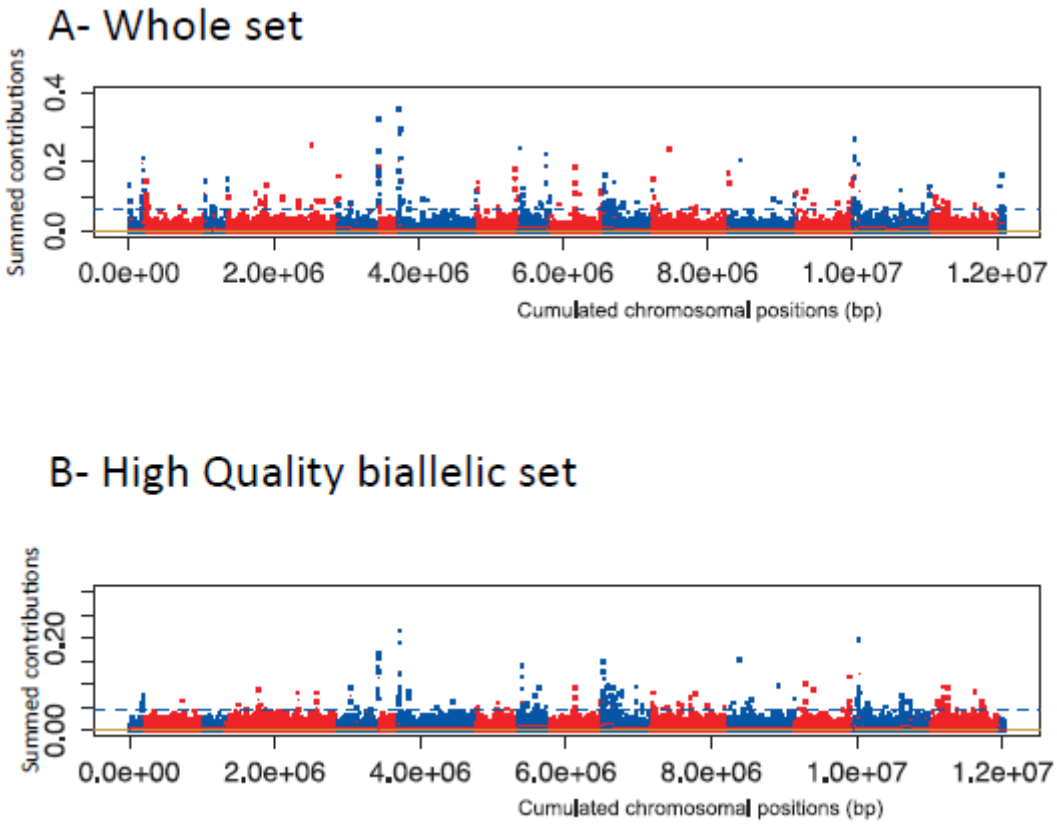


Figure 3A and B: The contribution to the 1st axis of PCA of SNPs contained in 500bp segments is plotted along the genome. A) Results are plotted for the whole set of variants. B) Results obtained for the set of high quality biallelic variants.

The second method aiming at detecting differences in the genome of the strains of the two populations is the Nucleotide diversity π. In this case, this parameter is calculted in sliding windows of 1kb and the genome scan of the values obtained from the set of high quality biallelic variant is presented in Fig. 4A. We could also select 1% regions having the highest values for π. The corresponding list of genes obtained from this test is given in table 5.

Table 5: Table of genes obtained with the different tests performed. Main genes involved in metal homeostasis are in black bold letters, and main genes involved in velum formation are in blue bold letters.

Method	Set of genes
PCA whole set of SNPs	*AI3, AI4, AI5_ALPHA, AQY2, ARN2, ARO10, BBC1, BI2, BI3, BI4, BSC1, BUD2, COA1, COB, COS10, COS6, COS7, COS8, COS9, COX1, COX2, CSM4, CYC8, DAN4, DBR1, DCI1, DPH2, EHD3, ENA1, FIT1,* ***FLO1, FLO11, FLO5****,* ***FRE3****, FZF1, HAP2, HKR1, HOL1, HPF1, HSP150, HXT3, HXT4, IMA1, IMD4, IME4, KDX1, KRS1, LAS17, LDB18, LOS1, LYP1, MDH2, MLH3, MLP2, MNN4, MNT2, MRL1, MRP8, MST1, NUM1, PAU11, PAU4, PAU6, PAU8, PDC1,* ***PIK1****, PIR3, PRM7, PRM9, PRP22, PWP1, Q0255, RPL40A, RPS30B, SDS22, SKM1, SKY1,* ***SLN1****,* ***SMF1****, SNR43, SNU114, SPT21, SUF11, SUP7, SVS1, tD(GUC)J2, THI7, TIR3, tR(UCU)J1, tS(AGA)D2, tW(CCA)P, UIP3, YAL067W-A, YAL068W-A, YAL069W, YAR023C, YAR028W, YAR029W, YAR1, YBL100W-A, YBL100W-B, YBL107W-A, YBL108W, YBL109W, YBL113C, YBT1, YCL076W, YCR108C, YDR261W-A, YDR261W-B, YDR379C-A, YDR543C, YER186C, YER187W, YER188C-A, YFL063W, YGL260W, YGL262W, YHL048C-A, YHR217C, YIL025C, YIL156W-A, YIL156W-B, YIL169C, YJR162C, YKL202W, YKL223W, YKL225W, YKR015C, YLL065W, YLL066W-B, YME1, YMR317W, YNL034W, YNL208W, YNL284C-A, YNL284C-B, YNL337W, YNL338W, YNR065C, YNR066C, YNR075C-A, YNR077C, YOL166C, YOL166W-A, YOR072W-B, YOR387C, YPS5, YPS6, YRA2,* ***ZRT1***
PCA High quality biallelic variant	*ABF1, AIM22, AIM34, ALG5,* ***ALR1****, AMD1, APC5, AQY2,* ***ARN2****, ARO10, ATG29, BSC5, BUD2, BUD22, CFT2, CGI121, CLP1, COS9, CRT10, CSM4, DAN4, DBR1, DCI1, DDC1, DNA2, DPH2, DYN1, ECM30, ENA1,* ***FRE6****, FYV12, FZF1, GAC1, HER1, HOL1, HRP1, HSP82, HXT4,* ***ICR1****, IMD4, IME4,* ***IRA1, IRA2****, KDX1, LAS17, LOS1, LSM5, LYP1, MCP1, MDH2, MET30, MLH3, MNT2, MRL1, MSD1, MSS1, MST1, NDI1, NMA1, NSE1, OPY2, OXP1, PAU7, PDC1,* ***PIK1****, PIR3, PRM9, PRP22, PWP1,* ***PWR1****, QDR1, QRI1, REC102, REV3,* ***RGA2****, RMD1, RPL16A, RPL40A, RPN7, RPS30B, RRT8, SCC4, SDH1, SEC24, SEC63, SGO1, SKM1, SKP2, SKY1,* ***SLN1****,* ***SMF1****, SMM1, SNU114, SPO20, SRY1,* ***STE7****, SUP7, SVS1, tD(GUC)J2, THI7,* ***TRE2****, TRM13, tS(AGA)D2, tT(UGU)P, UBI4, UIP3, UTP8, VHS2, YAR023C, YAR028W, YAR029W, YAR1, YDR274C, YDR379C-A, YER145C-A, YER186C, YER187W, YGL258W-A, YHR097C, YIG1, YIL025C, YIL058W, YJL045W, YJR146W, YKL162C, YKR015C, YLL056C, YLR149C, YME1, YML096W, YMR317W, YNL208W, YNL277W-A, YNR065C, YNR066C, YPL199C, YPR077C, YPR078C, YPR108W-A, YRA2, YTH1,* ***ZRT1***
Nucleotide diversity	*AIM22,* ***ALR1****, APC5, ATG29, BSC5, BUD2, CFT2, CLP1, COS9, CRT10,*

	CSM4, DBR1, DCI1, DPH2, EAF3, ECM30, ENA1, FYV12, FZF1, HAP4, HER1, HMS2, HSP82, ***ICR1****, IMD4, IME4, IRC23, IRR1, KDX1, LAS17, LOS1, LSM5, LYP1, MLH3, MLP2, MRL1, MSD1, MSS1, MST1, NSE1,* ***PIK1****, PIR3, PRM9, PRP22, PWP1,* ***PWR1****, QDR1, QRI1, REV3,* ***RGA2****, RMD1, RPL40a, RPS30b, RRT8, SCC4, SDH1, SET6, SGO1, SKM1, SKY1,* ***SLN1****,* ***SMF1****, SNU114, SRY1,* ***STE7****, SUP7, SVS1, tD(GUC)J2, THI7,* ***TRE2****, TRM13, tS(AGA)D2, UBC7, VMA1, VPS3, YAR023C, YAR028W, YAR029W, YAR1, YDL159W-A, YDR379C-A, YER145C-A, YER186C, YER187W, YIG1, YIL025C, YIL058W, YJL045W, YJR146W, YKL162C, YKL222C, YKL223W, YKR015C, YLR149C, YME1, YML096W, YMR317W, YNL208W, YNR066C, YOL047C, YOL098C, YOL099C, YPL199C, YPR077C, YPR078C, YRA2, YSN1****, ZRT1, ZRT3*
Tajima's D	*ADH4, AQY2,* ***ARN2****, ARO10, ARP1, BUD2, CLP1, CSE1, CSM4, DBR1, DCI1, DGA1, DOA1, DYN1, EGT2, ENB1, ENV9, FMP30, FMS1, FPS1, FRE6, FUR1, FYV12, GAP1, GCD14, GPB1, HAP2, HER1, HRP1, HSP82, IES3, IMD4,* ***IRA1****, ISR1, ISU2, KCC4, KDX1, LAS17, LOS1, LSM5, LYP1, LYS9, MCD1, MDM31, MLH3, MLP2, MNT2, MOT1, MRL1, MSD1, MSS1, NHP10, NRG1, OSW2, OXP1, PDR11, PDR15,* ***PIK1****, PRO2, RAD27, REV3, RGA2, RHO2, RPL40A, RPN7, RPS30B, RPS4A, RTC6, RTT10, RTT101, SCC4, SEG1, SER33, SET6, SHS1,* ***SLN1****,* ***SMF1****, SMM1, SNQ2, SNU114, SOG2, SPO22, SPT2, STB4,* ***STE7****, SUP7, SVS1, TAH1, tD(GUC)J2, TOP2, TPO5, tR(UCU)J1, TUM1, UBC7, UBI4, UGA1, URA1, VPS3, WHI2, YAR1, YCL022C, YDL159W-A, YDR379C-A, YER145C-A, YER186C, YER187W, YHL042W, YHR193C-A, YIG1, YIH1, YIL024C, YIL025C, YJL045W, YJR146W, YKL162C, YKR015C, YLR352W, YML096W, YMR090W, YNL018C, YNL089C, YNR014W, YPR077C, YPR078C, YRA1, YRA2, YTH1,* ***ZRT1***

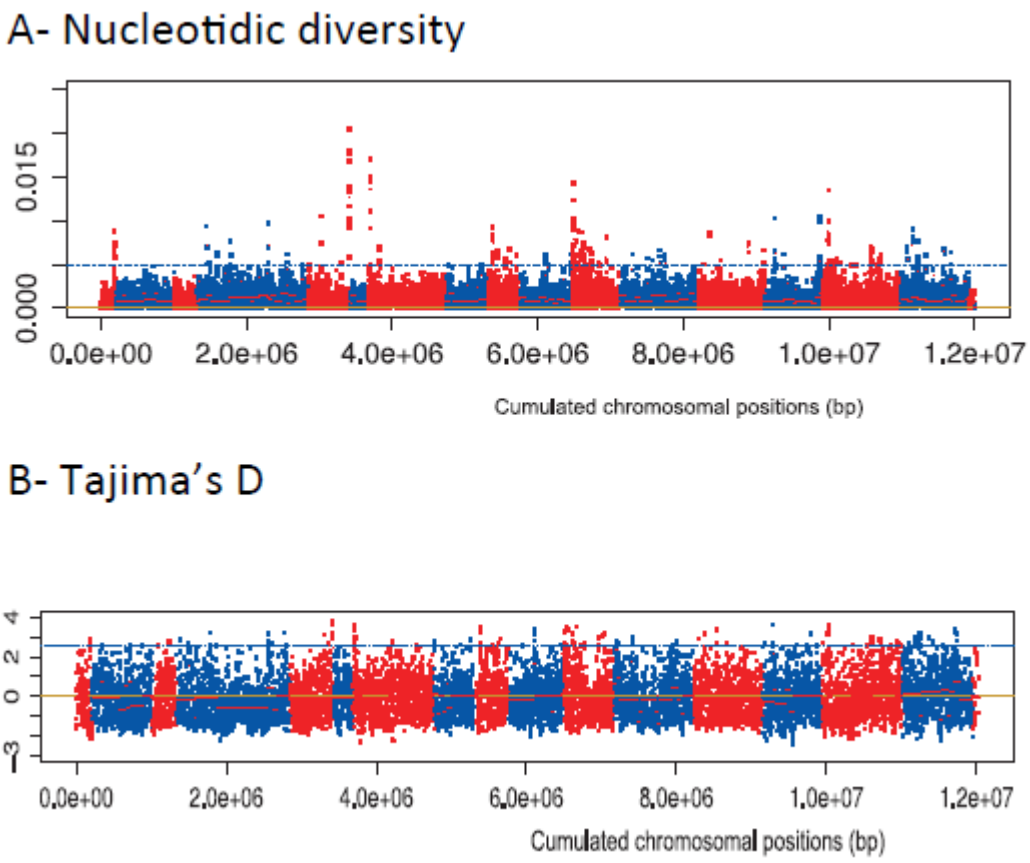


Figure 4: A: Genome-wide data obtained for Nucleotidic diversity π per 1kb sliding windows. Chromosomal coordinates are given in concatenated form, and chromosomal changes are materialized by color alternance. B: Genome-wide data obtained Tajima's D per 1kb sliding windows.

The third method, the variation of Tajima's D along the genome, is used here in order to detect possible balancing selection. The result of the genome scan of 1kb sliding windows given in Fig. 4B shows the wide dispersion of this statistic along the genome. We can hypothesize that this dispersal is related either to the population structure or to the lower recombination observed inside the group of flor strains, very likely caused by a lower rate of sexual reproduction. We also selected the set of 1% region, and the genes corresponding to these regions are given in table 5.

The comparison of these three lists based on the set of High quality variants with a Venn diagramm shows that 45 genes are shared by the three lists, and among them *PIK1*, *RGA2*, *SLN1*, *STE7*, members of the MAP kinase signaling cascade, *ZRT1* and *SMF1* encoding the two divalent metals transporters, and *HSP82*. Eighty five genes were obtained by at least 2 methods, such as *IRA1* coding for a negative regulator of Ras protein signal transduction, other genes involved in metal ions transport: *ARN2*, *FRE6*, *ALR1* and *SKY1* (encoding a

kinase involed in cation homeostasis), genes involved in protein degradation *UBI4* or *YME1*, and coding for the aquaporine *AQY2*.

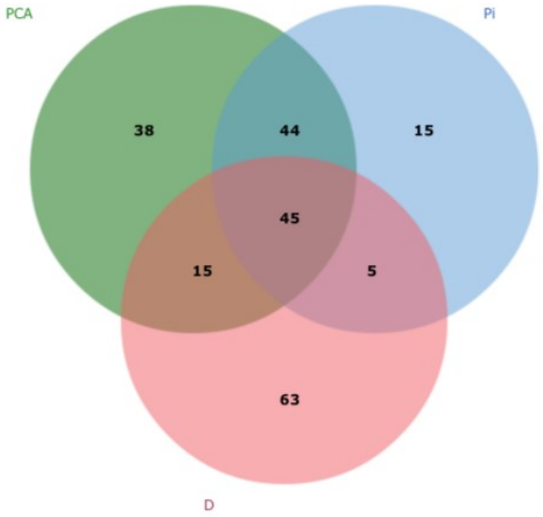


Figure 5: Venn Diagramm comparing the three set of genes obtained by PCA (PCA) nucleotidic diversity π or Tajima's D (D).

As any of the variants obtained here could be located in promoter regions or being synonymous, we searched for the impact of variants differentiating wine and flor strains that differ on protein structure. Twelve proteins were detected as truncated in the genome of flor strains: Vhs1, Aft1, Mnp1, Mmp1, Ctl1, Lto1, Ygl010w, Yar028w, Ydr010c, Yjr079w, Aqr1, Gpx2, the two latter being truncated of 7 and 6 amino acids only respectively. For four other proteins Pis1, Set2, Bna2, Rom1 the stop codon was replaced leading to a longer protein. Some of these genes have important function for cell: Vhs1 is a cytoplasmic serine/threonine protein kinase wich a potential role in G1/S phase progression, Aft1 is a transcription factor involved in iron utilization and homeostasis, Mnp1 is a protein associated with the mitochondrial nucleoid, which is required for normal respiratory growth, Mmp1 is a High-affinity S-methylmethionine permease, Ctl1 is a RNA 5'-triphosphatase, Lto1 is an essential protein that forms a complex with Rli1p and Yae1p, and its overexpression leads to the inhibition of pseudohyphal growth, whereas its deletion leads to filamentous growth (Jin, Dobry, McCown, & Kumar, 2008).

Comparison of P3-D5 flor and K1 wine yeast transcriptomes

In order to understand the phenotypic differences between wine and flor strains on a media enabling velum growth for flor strain, we compared their transcriptome. As wine strains do not form a velum, a shaken culture was chosen in oder to restrain the differences to the physiological ability of the strain, and limit differences caused by the specificities of velum growth. The expression of 1090 genes varied significantly with a log ratio greater 0.5 than two between the two strains (after eliminating ORFs differentially matching to the genome of each strain). Among them 564 and 536 genes were repressed and overexpressed respectively in the genome of P3-D5 in comparison to K1.

The Go Term categories for Biological processes indicates that many categories of genes are differentially expressed (table 6). Differences in carboxylic acid transport and metabolism, amino-acid transport, hexose transport, thiamine sysnthesis and transition ion metal transport were found. Indeed we could notice a higher expression of floculins (*FLO1,5,9,11*) hexose transporters (*HXT1, HXT2, HXT3, HXT4, HXT7*), genes encoding cytochrome subunits and other mitochondrial proteins (*GCV1*, *COX2*, *COB*, *AIM24*, *SUE1*, *CYC7*, *COX5B*) and thiamine sysnthesis genes in the genome of flor strains P3-D5. For some categories we could observe contrasted patterns as for amino acid uptake: *BAP3*, *BAP2*, *MUP1*, *TAT2*, *AGP1*, *MEP3*, *LST4*, *TNA1*, *SGE1*, *GNP1* were repressed and *PUT4, GAP1, DAL5, UGA4, SEO1, MMP1* were more expressed in the transcriptome of P3-D5. Divalent metal transporters present a contrasted pattern: *ZRT1* involved in zinc transport as well as *ZRT2*, *CUP1* and *CUP2* were up regulated whereas many genes involved in iron transport (*FRE1*, *ARN1*, *VHT1*, *SIT1*, *FRE3, FIT1*) as well as *SMF1* gene were repressed. However after checking for primer match, we could detect that the differences in hybridization of *ZRT1* could be associated to the poor hybridization of wine allele, but this was not the case for *ZRT2* or *FRE1, FRE2, FRE3.* Interestingly a search for publication enrichment made at YeastMine (http://yeastmine.yeastgenome.org/yeastmine/bag.do) gave significant associations with transcriptome analysis investigating mitochondrial dysfunction (Epstein et al., 2001) (pvalue 4.10^{-10}) analysis of the involvement of *BDF1* in salt stress response (Liu et al., 2007) (pvalue 3.10^{-9}), and zinc carency during fermentation (Cheraiti, Sauvage, & Salmon, 2008; Wu et al., 2008) (pvalue 1.10^{-8} and 3.10^{-3} respectively), and the highest similarity has been observed with a study of Daran-Lapugade et al. (Daran-Lapujade et al., 2004) who investigated the transcriptional changes related to

growth on glucose and maltose in comparison to ethanol and acetate. Comparison of flor and wine yeast transcriptomes suggests that flor yeast senses zinc deficiency and salt stress and possesses a higher mitochondria efficiency. Other significant differences between flor and wine yeasts were also detected, such as transcriptional regulation by inositol (Santiago & Mamoun, 2003), that indicates a response of flor yeast to a deficiency in inositol**.**

Aquaporins *AQY2* and *STL1* presented also opposed expression profile: expressed and repressed in flor strain P3-D5 in comparison to K1 respectively. Surprisingly *STE20* was repressed in P3-D5 transcriptome.

Table 6: Differentially expressed genes according to Go biological processes (Yeast mine, Pvalue 0.05, after Bonferroni Holmes correction. Only classes with more than 40 genes have been included)

GO Term (2062 categories)	Pvalue	Matches
single-organism process	8.88E-10	753
single-organism cellular process	1.13E-06	713
single-organism metabolic process	2.84E-09	441
small molecule metabolic process	1.25E-16	240
single-organism biosynthetic process	2.40E-09	208
organonitrogen compound metabolic process	8.83E-10	185
oxoacid metabolic process	3.05E-13	133
carboxylic acid metabolic process	1.20E-13	131
oxidation-reduction process	2.56E-09	128
organonitrogen compound biosynthetic process	1.78E-08	110
small molecule biosynthetic process	2.01E-09	102
cellular amino acid metabolic process	1.30E-08	92
response to chemical stimulus	0.011655	92
transmembrane transport	0.02412	86
lipid metabolic process	0.005794	76
ion transport	0.00868	72
cellular response to chemical stimulus	0.009461	71
organic acid biosynthetic process	3.16E-07	70
carboxylic acid biosynthetic process	3.16E-07	70
cellular lipid metabolic process	0.03468	63

alpha-amino acid metabolic process	4.69E-05	60
cellular amino acid biosynthetic process	6.07E-06	55
lipid biosynthetic process	0.026347	49
cofactor metabolic process	0.015687	47
organic hydroxy compound metabolic process	6.37E-04	45
anion transport	0.002292	45
monocarboxylic acid metabolic process	1.19E-04	43
alcohol metabolic process	9.92E-04	41

One unexpected feature was the overexpression of DNA helicases with telomerase recombination activity (*YRF1-1*/*2*/*3*/*4*/*5*/*6*/*7*).

Evaluation of the role of ZRT1 gene in wine fermentation/velum ageing adaptation

Given the frequency of transporters involved in divalent metals in the set of variant genes, we chose to study *ZRT1*, coding a high affinity zinc transporter. *ZRT1* is one of the gene for which we detected the highest divergence between wine, flor and S288c. The phylogenic tree of *ZRT1* (Fig. 6) shows clearly the high divergence between flor and wine allele: flor strains possess an allele of *ZRT1* similar to African or USA oak strains alleles suggesting an introgression from an African-like origin in flor strains or a specific divergence of wine alleles. However the two surrounding genes *ADH4*, a zinc dependent alcohol dehydrogenase, and *FZF1*, a transcription factor involved in the regulation of *SSU1*, 4671bp and 198bp distant of *ZRT1* respectively, carry two different alleles for flor and wine strains but these alleles are very close, and clearly different from alleles from strains isolated from other origins such those of African palm wine strains USA oak strain (Fig. 7). The phylogeny observed for *ADH4* and *FZF1*, is also in agreement with the phylogeny obtained for the whole genome. The *S. paradoxus* allele of *ZRT1*, is close to the Malaysian branch of *ZRT1* tree (unshown data), and the value of a McDonald Kreitman test (NI=4.4, pvalue = 0.000) (McDonald & Kreitman, 1991), indicate a balancing selection for that gene which has also been recently shown (Engle and Fay 2013). The differences in the topology of the trees between *ZRT1* and its neighbouring genes *ADH4* and *FZF1* suggest that flor strains have recovered a version of *ZRT1* close to the African or USA oak alleles. This

suggests also that wine allele is either a loss of function allele, or an allele with unfavourable properties in flor environment, which has been replaced with a more suited form for flor environment, and gives another clue of the potential role of this allele of *ZRT1* in flor yeast.

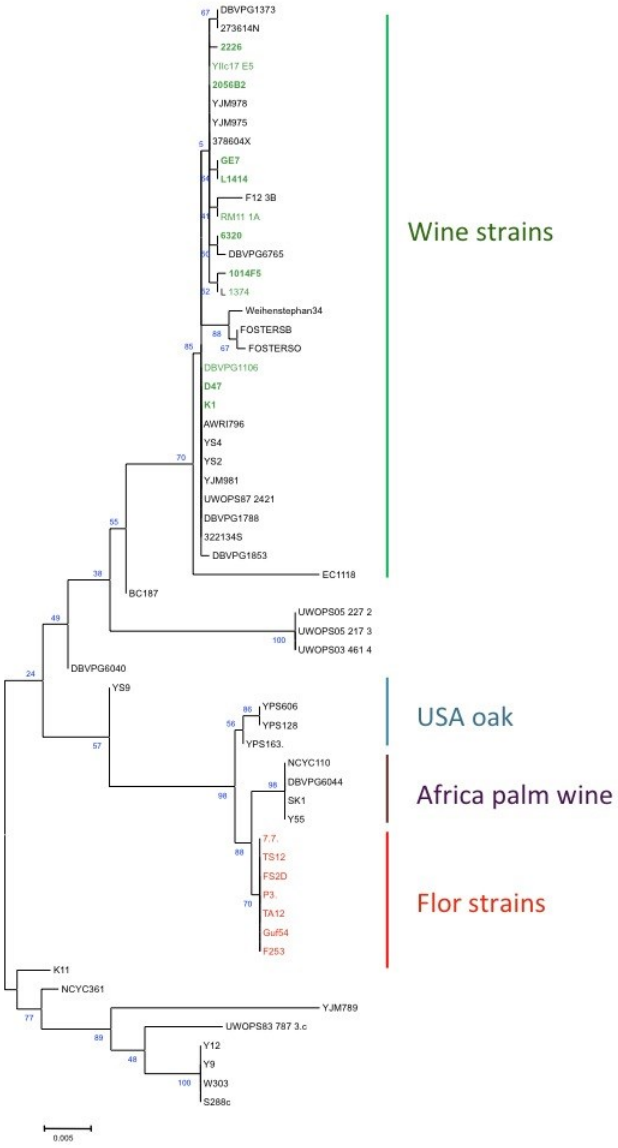


Figure 6: Neighbor joining tree presenting the phylogeny of *ZRT1*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) using 59 nucleotide sequences from the available genome sequence.

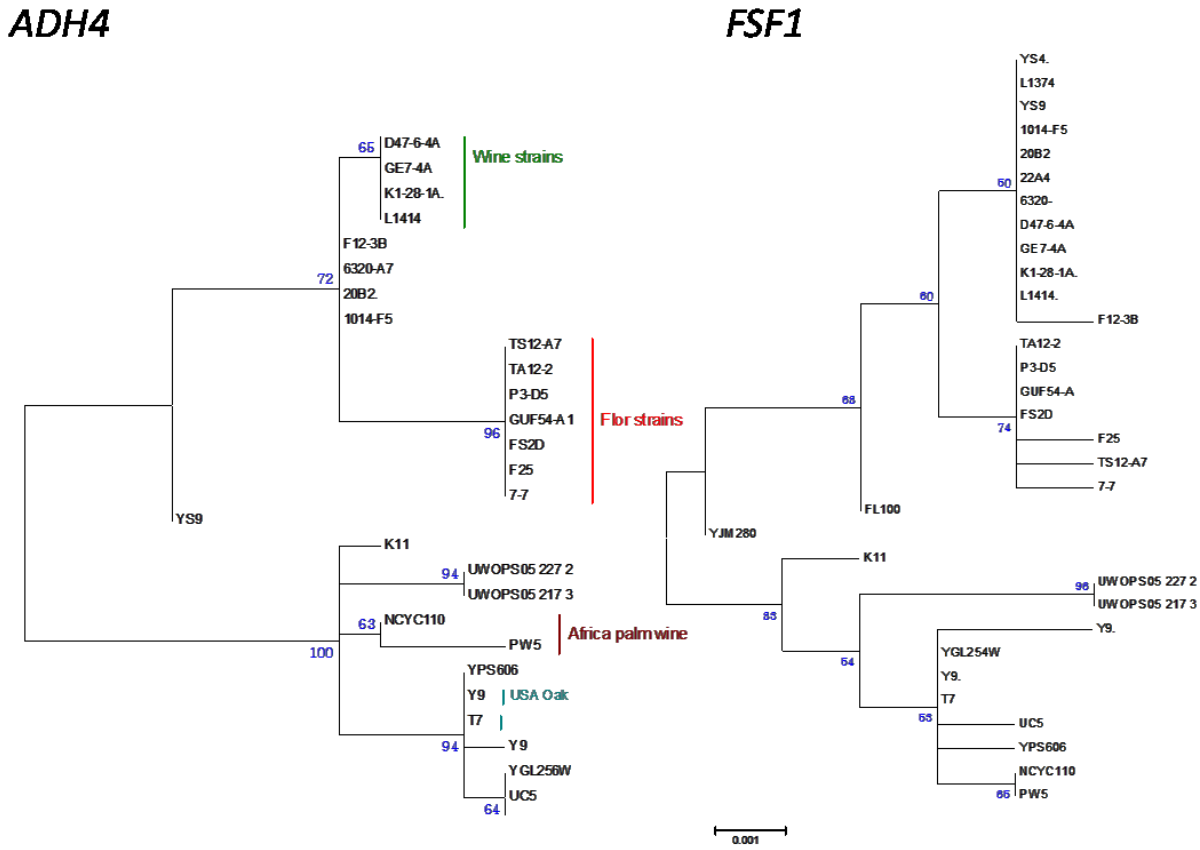


Figure 7: Neighbor joining tree presenting the phylogeny of *ADH4* and *FZF1*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) using 30 and 32 nucleotide sequences (*ADH4*) and (*FZF1*) respectively from the available genome sequence.

Zinc is an essential oligoelement for eukaryotic cell since it is a cofactor of many proteins and enzymes such as *SOD1*, the Cu/Zn-superoxide dismutase. As no difference has been seen on fermentation kinetics on grape must after allelic exchange of *ZRT1* (Engle and Fay, 2013), we hypothesized that the allele of *ZRT1* could lead to phenotypic differences in the environment of biological ageing and, as cells are exposed to oxidative stress, to a possible difference in antioxidant activity. To evaluate the role of these mutations, we performed an allelic replacement of the *ZRT1* flor allele in wine strains and of the wine allele in flor strains.

A spot test assay was performed for the evaluation of oxidative stress resistance of *ZRT1* wild-type and mutant strains. Cells were spotted on YEPD, YEPD + 6 mM H_2O_2 and YEPD + 2 mM H_2O_2 + 1 mM EDTA plates (Fig. 8). On the contrary to the expected, flor strains displayed less resistance to oxidative stress than wine strains (Fig. 8B). We could

not detect any differences in the growth rates of strains bearing *ZRT1* wine allele or flor allele: both alleles displayed a similar activity under our test conditions, at normal and low zinc conditions. Liquid culture did not reveal any differences as well.

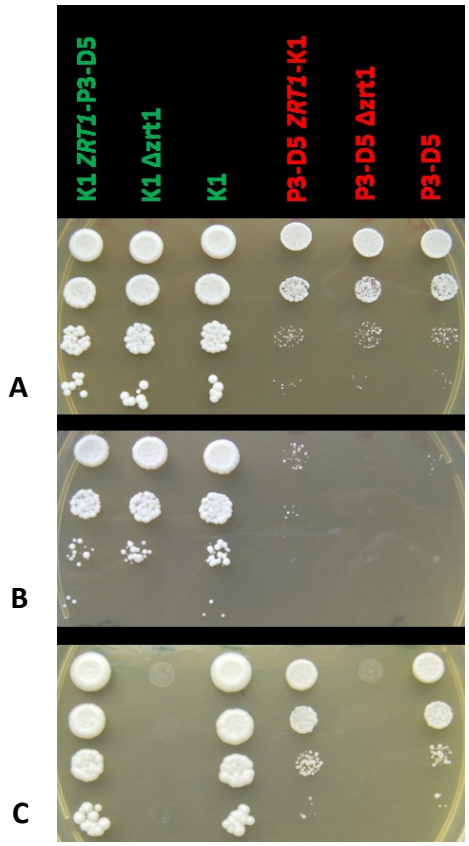


Figure 8: Oxidative stress resistance assay. A: YEPD. B: YEPD + 6 mM H_2O_2. C: YEPD + 2 mM H_2O_2 + 1 mM EDTA. Flor strains are represented in red, wine strains in green. Experiment was carried out in triplicate.

The same test was performed on YNB media (YNB w/o amino acids + 1mM EDTA + 2% agar pH 4.2 (Zhao and Eide, 1997)) (Fig. 9), and again no difference in the growth of the strains was observed.

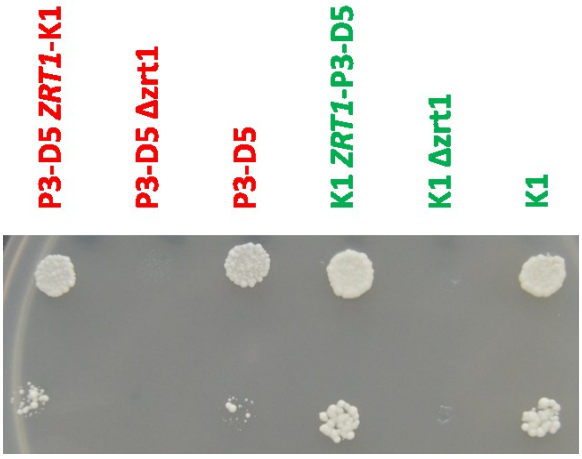


Figure 9: Spot-assay on YNB + 1 mM EDTA pH 4.2. Flor strains are represented in red, wine strains in green. Experiment was carried out in triplicate.

An alternative possibility was the growth media too distant from wine. We thus compared the growth of the different strains on a flor synthetic medium containing 8% ethanol + 10X vitamins without $ZnSO_4$ (Fig. 10). Once again, the different alleles did not show different behavior under low zinc conditions. Even deleted mutants displayed the same phenotype as the parental strain and did not seem to suffer from the zinc limiting concentration in the medium. This discrepancy with plate experiments could be explained with the fact that low zinc solid medium contained EDTA, which chelates divalent cations, not only zinc, and the activity of *ZRT1* could as well be related to Fe^{2+}, Cu^{2+} or Mn^{2+}, or Mg^{2+}.

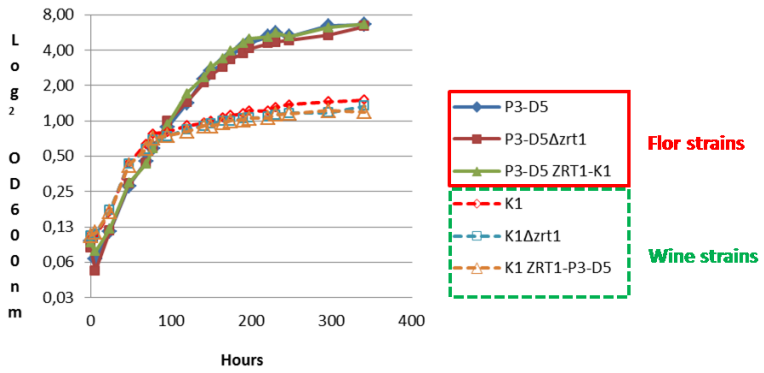


Figure 10: Growth curve on flor medium 8% ethanol + 10X vitamins without $ZnSO_4$. Experiment was carried out without replicate.

We also evaluated biofilm forming ability of wild-type and mutant flor strains under zinc limiting and stress oxidative conditions (Fig. 11 A and B). With the progressive increase of EDTA and hydrogen peroxide concentrations, velum formed was progressively thinner till arrive to complete inability of strains to produce biofilm. No difference was observed between different strains in both conditions.

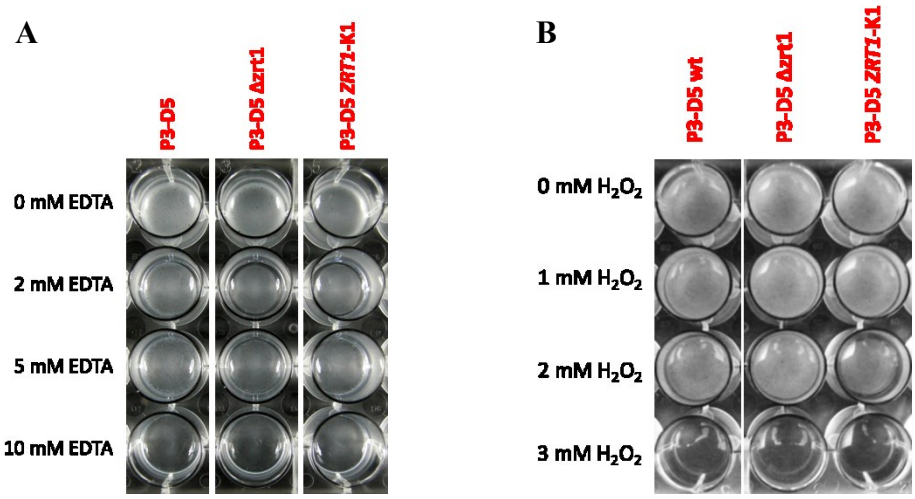


Figure 11: Biofilm forming ability of *ZRT1* mutant flor strains under zinc limiting (A) and oxidative stress conditions (B). Experiments were carried out in triplicate.

Discussion

Flor strains phylogeny

In order to detect genome variations involved in adaptation of flor strains to biological ageing conditions we sequenced the genome of 10 flor strains and 8 wine strains. The phylogenic tree obtained (Fig. 1) is globally in agreement with what previously described by Liti et al. (2009) with several clusters (European/Wine, Sake, Oak, Malaysian Bertram palm, African palm wine) but flor strains were contained in a new cluster close to wine strains (Fig. 1B). Population inference with admixture or DAPC revealed 9 populations, and the same number of clusters was found in a recent study exploring *S. cerevisiae* diversity from a large collection of 267 strains, isolated from a wide range of geographical locations and environmental niches with RAD-tag sequencing (Cromie et al., 2013). In this last study, two groups of strains were very close to wine strains: a group of olive isolates not differentiated from wine strains, and a group of clearly differentiated vineyard isolates from New Zealand, that may be also related to the flor yeast group and would require

comparison. The group of flor strains represents a separate lineage from wine strains, and the mean nucleotide divergence between flor strains is 60% of that calculated for wine strains. We can make the hypothesis that the expansion of this group is more recent than that of wine strains, even though we cannot rule out that these differences are originated from different life styles leading to different growth speed. The slight differentiation between the two subgroups, Hungarian and French strains, or Spanish and Italian strains that we could call "central Europe" and "Mediterranean" respectively, suggests few exchanges between the two groups. The basal position in the tree of a Lebanese flor genotype was not confirmed by the population structure analysis, which revealed that this strain has a mixed genome between wine and flor genomes. The industrial strains EC1118, 59A, QA23 and VIN13 located close to flor strains, appeared also as a mosaic genome between flor and wine strains. This can be seen as well from principal component analysis obtained on the whole set of SNPs for the differential characterization of flor and wine strains.

Search for regions explaining phenotypic differences between wine and flor

We used three different approaches to detect regions possibly involved in wine and flor differentiation: a Principal Component Analysis, a genomescan of Nucleotide diversity π, and of Tajima's D. Among the regions identified by these techniques, we found only 19 genes (*FLO11, HAP2, IMA1, MDH2, MST1, PIR3, SPT21, YME1, ALG5, FYV12, IRA2, MSD1, SGO1, HAP4, VPS3, ENV9, FPS1, MDM31, RTC6*) shared with the set of 688 genes recently described as important for film forming (Ryan et al., 2012). *FLO11* and non-coding RNA involved in its regulation *ICR1* and *PWR1*, that were also detected, were expected given their key role in biofilm formation (Reynolds and Fink, 2001; Zara et al., 2005; Fidalgo et al., 2006; Bumgarner et al., 2009). We detected also 2 other genes, *FLO1* and *FLO5*, encoding two lectins that recognize mannose oligomers with a Ca^{2+} binding motif (Brückner and Mösch, 2012). In contrast we did not noticed major differences in the *FLO10* gene, despite its resemblance with *FLO11*. The presence of other genes encoding cell-wall proteins in the whole set of variants was also expected, as these genes are enriched in intragenic repeats, which are unstable. The size variation of the repeats can generate functional variability, resulting in quantitative alterations in phenotypes such as adhesion, flocculation or biofilm formation (Verstrepen et al., 2005).

Another major finding is the number of genes which are associated to the regulation of *FLO11*: *IRA1* and *IRA2* for the Ras/cAMP/PKA signalling pathway, and *HKR1*, *SLN1*, *RGA2*, *STE7* for the MAP kinase signalling pathways. Recently Kvitek and Sherlock observed that adaptation to low glucose culture lead to a high frequency of mutations in the Ras/cAMP/PKA pathway and the high osmolarity glycerol (HOG) response pathway (Kvitek and Sherlock, 2013). *SFL1*, which encodes a repressor of *FLO11*, was also detected as variant (although its rank was slightly lower than the 1% top variants) but this gene contains also many variations between wine and flor strains, and we noticed a frameshift in its C terminal part for two flor yeasts from Jura (P3-D5) and Hungary (TA12). Interestingly, these two strains produce a thin velum, but do not have the 111bp deletion in non-coding RNA *ICR1* (Fidalgo et al, 2006). Another frameshift was also detected in gene *LTO1* which deletion in strain Sigma1278b leads to an increase in filamentous growth.

The comparative transcriptome analysis of wine and flor strains gives complementary insights into the genetic specificity of flor yeasts. Flor strain P3-D5 has as expected a higher expression of *FLO11*, but also of other flocculins genes (*FLO9*, *FLO5*, *FLO1*), that we detected as remarkably polymorph from our data set containing all variants. We could detect as well a major up-regulation of hexose transporters of flor yeast, when *HXT3* and *HXT4* appeared as variant, of the subunits of cytochrome genes (*COX1,2,3,5,715, COX5B, COB*) when *COX2* and *COB* genes were also detected polymorph. Comparison with other transcriptomic data (proposed by data mining with YeastMine) suggested a better efficiency of flor yeast mitochondria that is in agreement with their lifestyle, but revealed as well that flor, more than wine yeast, suffer more from a lack of zinc, as well as salt osmotic stress. In addition flor yeast apparently would display higher requirement in inositol.

The down regulation of many genes involved in iron uptake (*FRE1*, *ARN1*, *VHT1*, *SIT1*, *FRE3*) may be caused by the Aft1 truncated protein or to the higher requirement of iron. Another unexpected feature was the induction of DNA helicases with telomerase recombination activity (*YRF1-1*/*2*/*3*/*4*/*5*/*6*/*7*) in the genome of flor yeast suggesting telomerase defects.

Evaluation of the role of ZRT1 gene in wine fermentation/velum ageing adaptation

Unfortunately, despite a large divergence between wine and flor alleles, we were not able to find a phenotype associated to *ZRT1* genetic variation that may explain an adaptive evolution of flor strains. However, we found numerous variant genes involved in divalent metals transport, such as *ZRT3* (the vacuolar transporter of zinc, whose induction is activated under low zinc conditions), *SMF1* and *ALR1*, coding for manganese and magnesium transporters respectively, *TRE2* that functions with Tre1p to regulate ubiquitylation and vacuolar degradation of Smf1p, or *UBC7* which is involved in resistance to cadmium poisoning, indicating that metal homeostasis is important for flor strains. The comparison of P3-D5 flor and wine yeast K1 transcriptome stresses this point with the up regulation of genes involved in zinc import suggesting a deficiency in zinc, and the repression of genes involved in iron homeostasis.

Zinc is an essential nutrient of all organisms since it plays a structural role in many proteins and is also a catalytic component of over 300 enzymes, such as superoxide dismutase (Vallee and Auld, 1990). Cu/Zn-superoxide dismutase, encoded by the gene *SOD1*, has an important antioxidant role since it's used by aerobic organisms and some higher eukaryotes against the damages caused by reactive oxygen species (ROS) to nucleic acids, proteins, lipids and other cellular components. In *Candida albicans ZRT1* has an essential role for pathogenicity as it allows zinc absorption from the host (Citiulo et al., 2012). These authors found the role of a new gene *PRA1* coding for a protein chelating zinc, that they called zincophore, by analogy with siderophores. This gene is very well conserved among ascomycetes, and is present in *S. cerevisiae* genome. Because flor yeasts during biological ageing have an aerobic metabolism and in aerobic cells the mitochondrial respiratory chain is the main source of ROS (Cadenas, 1989), *ZRT1* variation may have contributed to antioxidant defense of flor yeast. However, we could not confirm this hypothesis. Surprisingly, wine strains appeared most resistant to oxidation than flor strains. Even wine yeasts reactive oxygen species can be produced during fermentation (Landolfo et al., 2008; Rosenfeld and Beauvoit, 2003), the mitochondrial respiratory chain is the main source of ROS (Cadenas, 1989) and it is not clear why flor strains are poorly resistant.

One key role is very likely the involvement of zinc availability in phosphatidylinositols synthesis (Henry et al., 2012) as it has been as well seen recently (Guillas et al., 2013) that phosphatidylinositol (4,5)P_2 synthesized by Mss4 is necessary for Flo11 expression. Other

studies, failed to detect functional differences among different *ZRT1* alleles during fermentation (Engle and Fay, 2013). However, some authors reported that *ZRT1* not only has the function of carrying zinc inside the cell but could also influence the transport of other metals, such as cadmium (Gitan et al., 1998; Gomes et al., 2002; Gitan et al., 2003). Our data showing a phenotype of *zrt1* mutant in low zinc solid medium containing EDTA and the absence of phenotype in conditions without chelating agents suggests that the activity of *ZRT1* could be related to other metals. Finally, other proteins such as Vel1, that might have a role in velum formation (SGD http://www.yeastgenome.org/) and the product of its paralog Yor387c were identified as molecular markers of zinc deficiency as they are induced in response to zinc shortage by Zap1, the transcriptional factor that activates *ZRT1* transcription (Higgins et al., 2003). Therefore, further work is needed to better characterize the impact of *ZRT1* alleles.

The numerous mutations and translocations also found in *FRE2*, *FRE3* genes the mutations found in *FRE6* (chapter 4), and a frameshift leading to a truncated protein of Aft1 which may explain the lower expression of genes of the iron regulon, again makes the importance of ion homeostasis more critical, in flor yeasts.

Our data also highlight more variant genes that may have an impact on flor yeast phenotype. Among them, *HSP82*, one of the variant identified plays a role in resistance to acetaldehyde (Aranda et al., 2002), a compound formed at high level during flor ageing. Another highly interesting variant is *HXT3*, which carries mutations previously shown to confer a higher affinity for fructose transport (Guillaume et al, 2007).

In conclusion, the study and the comparison of the genome sequence of flor yeast with wine yeast enabled us to characterize the phylogenic and genomic specificities of flor yeast. We identified several regions possibly involved in the adaptation of flor yeasts. The detection of genes coding cell-wall proteins or involved in the regulation of filamentous growth could have been expected. In contrast, the identification of numerous genes involved in metal homeostasis is unforeseen and suggests that metals, and especially zinc, could be important during biological ageing. Still one of the major points brought to light by this work is the accumulation of mutations in specific genes creating a pattern of convergent mutations in regulatory networks, as seen from the multiple genes mutated in the pseudohyphal growth and HOG MAP kinase pathways, as well as in the different

regulatory pathways enabling fine tuning of *FLO11* expression. The deregulation of these regulatory networks is clearly one of the hallmarks of domestication on flor yeast genome.

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3. ANALYSIS OF OTHER VARIANT ALLELES POTENTIALLY RELATED TO FLOR DOMESTICATION

In addition to the evaluation of the allelic variants described in chapter 3, we report here a detailed study on several other variant genes: *FRE2*, *FRE3*, *PIK1* and *PDC1*. We will describe the variations observed for these genes and our efforts to identify a potential effect of these allelic variations during wine fermentation or flor ageing conditions. In addition, we present additional work performed for the detection of *ZRT1* activity.

4.1 Materials and methods

4.1.1 Yeast strains and media

Yeast strains used and their origin are listed in table 1.

Strain	Group	Genotype	Origin
2D MATa	Flor	MAT**a**; YDL227c::kanMX4	This work
2D MATα	Flor	MATα; YDL227c::kanMX4	This work
P3-D5 MATa	Flor	MAT**a** ; ho::loxP	This work
P3-D5 MATα	Flor	MATα; ho::loxP	This work
K1 MATa	Wine	MAT**a**; ho::loxP	This work
K1 MATα	Wine	MATα; ho::loxP	This work
P3-D5 Δfre2	Flor	MATα; ho::loxP; YKL220C::loxP-kanMX-loxP	This work
K1 Δfre2	Wine	MAT**a**; ho::loxP; YKL220C::loxP-kanMX-loxP	This work
K1 Δfre3	Wine	MAT**a**; ho::loxP ; YOR381W::loxP-ble-loxP	This work
K1 Δfre2,3	Wine	MAT**a**; ho::loxP; YKL220C::loxP-kanMX-loxP; YOR381W::loxP-ble-loxP	This work
K1 Δzrt1	Wine	MAT**a**; ho::loxP ; YGL255W::loxP-kanMX-loxP	This work
P3-D5 Δzrt1	Flor	MATα; ho::loxP ; YGL255W::loxP-kanMX-loxP	This work
P3-D5 *ZRT1*-K1	Flor	MATα; ho::loxP ; YGL255W:: YGL255W (K1)	This work
K1 *ZRT1*-P3-D5	Wine	MAT**a** ; ho::loxP ; YGL255W:: YGL255W (P3-D5)	This work
CEN.PK111-61A	Wine	MAT**a;** pdc1 (-6,-2)::loxP; pdc5(-6,-2) ::loxP ; pdc6(-6,-2)::loxP ura3-52	TUDelft Marijike Luttik; The Netherlands
CEN.PK Δpdc5,6 *PDC1*-2D	Wine	MAT**a**; pdc5(-6,-2)::loxP; pdc6(-6,-2)::loxP ura3-52; YLR044C::YLR044C (2D)	This work
CEN.PK Δpdc5,6 *PDC1*-K1	Wine	MAT**a;** pdc5(-6,-2)::loxP; pdc6(-6,-2)::loxP ura3-52; YLR044C::YLR044C(K1)	This work
K1 Δpik1	Wine	MAT**a**; ho::loxP; YNL267W::loxP-ble-loxP	This work
K1 *PIK1* Kan	Wine	MAT**a**; ho::loxP; pPIK1::pPIK1-loxP-kanMX-loxP	This work
P3-D5 Δpik1	Flor	MATα; ho::loxP; YNL267W::loxP-ble-loxP	This work
P3-D5 *PIK1* Kan	Flor	MATα; ho::loxP; pPIK1::pPIK1-loxP-kanMX-loxP	This work

PDC1 growth phenotype was evaluated on the synthetic flor medium 8% ethanol (flor medium composition reported in chapter 2) containing 10 times more vitamins for aerobic cultivation, and the synthetic must MS 300 for fermentations (modified version from Bely et al., 1990, where 200 g/L glucose were replaced with 100 g/L glucose and 100 g/L fructose, and 10 mg/L $FeCl_3$ were added).

4.1.2 Construction of mutants

Primers used to obtain *FRE* mutant strains were: FRE3BleFw and FRE3BleRv for *FRE3* deletion, FRE3verFw and FRE3verRv to confirm *FRE3* deletion, FRE2delFw and FRE2delRv for *FRE2* deletion, FRE2verFw and FRE2verRv to verify *FRE2* deletion. Selection of *FRE3* deleted strains was done on YEPD + phleomycin (150 µg/ml), of *FRE2* deleted strains on YEPD + geneticin (200 µg/ml) and of *fre2,3*Δ strains on YEPD + phleomycin (150 µg/ml) + geneticin (200 µg/ml). To construct *PIK1* mutants we deleted the most mutated part of the promoter, a 209 bp region, inserting a loxP-P_{AgTEF1}**-ble-**T_{AgTEF1}-loxP cassette and selecting transformants on YEPD + phleomycin petri dishes. To carry out the allelic replacement we inserted a loxP-kanMX-loxP cassette before the promoter portion that we wanted to replace, the amplification of this part of the promoter region fused with the kanMX cassette will allow us to carry out the allelic replacement. To delete part of *PIK1* promoter region we used PIK1delwFw and PIK1delwRv primers for wine strain and PIK1delfFw, PIK1delfRv for flor strains. Primers for KanMX insertion were: PIK1kanFw, PIK1kanwRv and PIK1kanfRv. *PIK1* deletion and kanamycin insertion we verified using the following primers: PIK1verFw, PIK1verRv, KanP1 and KanP2. Allelic replacement of *PDC1* flor and wine alleles was done in the strain CEN.PK *pdc1,5,6Δ* (MATa *pdc1*(-6,-2)::loxP; *pdc5*(-6,-2)::loxP; *pdc6*(-6,-2)::loxP ura3-52). This strain cannot grow on glucose and was cultivated on a particular medium (yeast extract 1%, peptone 2%, glycerol 2% and ethanol 2%). Primers used were: PDC1alldefFw and PDC1alldefRv to amplify allele, PDC1verdefFw and PDC1verdefRv to verify allelic replacement. As the triple deleted *pdc1,5,6Δ* mutant was unable to grow on glucose, the transformants were directly selected on YEPD. All the primers used are listed in table 2. The construction of *ZRT1* mutants is reported in chapter 3. PCR condition, reaction mix and protocol of transformation were the same used for *HO* gene deletion (see chapter 2).

Table 2: List of primers used

Primer	Sequence	Purpose
FRE2delFw	5' ACAAAAAACCATGCGACACACTACGACTTTCA CAAATTCTTCGTACGCTGCAGGTCGAC 3'	Obtaining *FRE2*::KAN insertion cassette
FRE2delRv	5' GTACTGTTTCCTTGCTTTTCTGAATTCTTTTCTG ATGAACGCATAGGCCACTAGTGGATCTG 3'	Obtaining *FRE2*::KAN insertion cassette
FRE2verFw	5' TCTTCCTTGCATCTGTGT 3'	Confirming *FRE2*::KAN insertion cassette
FRE2verRv	5' GGTTTACAGGAAAACAAGTAA 3'	Confirming *FRE2*::KAN insertion cassette
FRE3BleFw	5' AGCTACTGATTCGTTGGCGATGAAAGACTCT TTTTTGGTACGCTGCAGGTCGACAAC 3'	Obtaining *FRE3*::Ble insertion cassette
FRE3BleRv	5' GGAGGAAGAGAAATTTAAGTCATGGAACAAG GTGGGAGACTATAGGGAGACCGGCAGA 3'	Obtaining *FRE3*::Ble insertion cassette
FRE3verFw	5' TTTACACTGCGAACGGGATA 3'	Confirming *FRE3*::Ble insertion cassette
FRE3verRv	5' CAGAATTCGGAAAGAAGATGG 3'	Confirming *FRE3*::Ble insertion cassette
PDC1alldefFw	5' TCCATGGTAAGTGACAGTGCA 3'	Amplifying *PDC1* allele
PDC1alldefRv	5' GTGATGGCACATTTTTGCA 3'	Amplifying *PDC1* allele
PDC1verdefFw	5' GGAAGACATCTTTTCCAACGA 3'	Confirming *PDC1* allelic replacement
PDC1verdefRv	5' ATAATATTGTCCGCTGCCCCT 3'	Confirming *PDC1* allelic replacement
PIK1delwFw	5'AATTTATGGGAGAAATTCTGGGCTGCTGTTGCATA GTCAGTACGCTGCAGGTCGACAAC 3'	Obtaining of *PIK1*::Ble cassette from pUG66 in wine strain
PIK1delwRv	5' GACCCTGTTATACACGCTATCAAGTACGTAAA GGATAATACTATAGGGAGACCGGCAGA 3'	Obtaining of *PIK1*::Ble cassette from pUG66 in wine strain
PIK1delfFw	5' AATTTATGGGAGAAATTCTGGGCTGCTGTTGC ATAATCAGTACGCTGCAGGTCGACAAC 3'	Obtaining of *PIK1*::Ble cassette from pUG66 in flor strain
PIK1delfRv	5' GACCCTGTTATACACGCTATTCAGTACGTAAA AGATAATACTATAGGGAGACCGGCAGA 3'	Obtaining of *PIK1*::Ble cassette from pUG66 in flor strain
PIK1KanFw	5' CTAAGAATTTATGGGAGAAATTCTGGGCTGCT GTTGCATAGTTCGTACGCTGCAGGTCGAC 3'	Obtaining *PIK1*::KAN insertion cassette
PIK1KanwRv	5' TTTTTTTCTATTTTGAAGGCATGCAAGAGGTTC TGTGACGCATAGGCCACTAGTGGATCTG 3'	Obtaining *PIK1*::KAN insertion cassette in wine strains
PIK1KanfRv	5' TTTTTTTCTATTTTAAAGGCATGCAAGAGGCTT TGTGAGCATAGGCCACTAGTGGATCTG 3'	Obtaining *PIK1*::KAN insertion cassette in flor strains
PIK1verFw	5' CGACAGAAGAGAAATCGAAGC 3'	Confirming *PIK1*::Ble and *PIK1*::KAN insertion
PIK1verRv	5' TTTTCGGAGTGTTTGCACAG 3'	Confirming *PIK1*::Ble and *PIK1*::KAN insertion

4.1.3 Evaluation of ZRT1 phenotype using Phenotype Microarray analysis

Phenotype Microarray (PM) technology uses tetrazolium violet reduction as a reporter of active cellular metabolism (Bochner et al., 2001; 2008). The reduction of the dye causes the formation of a purple color, which is recorded by a CCD camera every 15 minutes and provides quantitative and kinetic information about the response of the cells in the PM plates (Bochner et al., 2001). The data obtained are stored in computer files and can be analyzed to compare the PM kinetics of different strains (Koo et al., 2004; Winterberg et al., 2005).

The strains were tested by the PM approach for 90 different carbon sources (PM1 and PM2A), 95 nitrogen sources (PM3), osmolarity (PM9) and pH gradient (PM10). The concentrations dispensed into the wells are Biolog patent unknown to the users. In order to

perform PM experiments the strains were grown overnight at 28°C on BUY agar (Biolog) and then cells were picked up with a sterile cotton swab and suspended in 15 ml of sterile water. Cell density was adjusted to 62% transmittance (T) on a Biolog turbidimeter. Inoculation fluids were prepared as follows: for PM1-2A, IFY-0 Base 1x supplemented with L-glutamic acid monosodium 5 mM, potassium phosphate (pH 6.0) 5 mM, sodium sulfate 2 mM, Dye Mix D 1x; for PM3, IFY-0 Base 1 supplemented with D-glucose 20 mM, potassium phosphate (pH 6.0) 5 mM, sodium sulfate 2 mM, Dye Mix D 1x; for PM9 IFY-0 Base 1x supplemented with D-glucose 100 mM, L-glutamic acid monosodium 5 mM, potassium phosphate (pH 6.0) 5 mM, sodium sulfate 2 mM, Dye Mix D 1x; for PM10, SC medium 1x supplemented with D-glucose 100 mM and Dye Mix E 1x. SC medium 1.2x was prepared mixing YNB w/o amino acids and ammonium sulfate (cat. no.Y1251, SIGMA) 8.04 g/L and Drop Out Mix Complete (cat. no. D9515, US Biological) 2.4 g/L, and sterilized by filtration. Cells suspension at 62% T were diluted in inoculation fluids adding 0.25 ml in12 final volume for each plate (0.5 ml in a final volume of 24 ml for PM1, 2A).

All PM plates were incubated at 28°C in an Omnilog Reader (Biolog Inc.) and monitored automatically every 15 min for 96 h for color development. To identify phenotypes gained or lost by strains, the kinetic curves obtained were compared using Omnilog-PM software (release OM_PM_109M).

4.1.4 Evaluation of PDC1 phenotype

Growth and metabolic production of allelic replacement mutants were monitored during aerobic cultivation on synthetic flor medium 8% ethanol + 10X vitamins and during fermentation on synthetic must MS 300 (modified version from Bely et al., 1990, where 200 g/L glucose were replaced with 100 g/L glucose and 100 g/L fructose, and 10 mg/L $FeCl_3$ were added). For aerobic cultivation strains were precultivated on YEPD, after 16 hours, and inoculated in YEPD + 5% ethanol. After 9 hours strains were washed twice, resuspended in synthetic flor medium 8% ethanol + 10X vitamins and inoculated (OD_{600} = 0.1) in 100 ml flor medium. Yeasts were cultivated for 300 hours at 28°C with agitation (180 rpm), in 500 ml flasks with foam cap. As strains were auxotrophic (MATa; *pdc5*(-6,-2)::loxP; *pdc6*(-6,-2)::loxP ura3-52; YLR044C::YLR044C-2D) 0.05 mg/ml uracil were added. For fermentations, strains were precultivated on YEPD and, after 16 hours, $2x10^6$ cells/ml were inoculated in 40 ml synthetic must MS 300 with the addition of uracil, as

previously described for aerobic cultures, and incubated at 28°C and 180 rpm agitation in 250 ml flasks. After 24 hours 10^6 cells/ml were inoculated in fermenters containing 1,1L synthetic must MS 300 + uracil.

Acetoin and 2,3-butanediol production were evaluated using gas chromatography (Agilent 6890) after chloroform liquid-liquid extraction from frozen supernatants. Briefly: in 15 ml pyrex tubes with screw cap were subsequently added 1 ml internal standard (1 ml hexanol diluted in 1L of 10% ethanol), 1 ml sample and 2,5 g potassium carbonate. After vortexing for a few seconds 2 ml chloroform were added in every tube and tubes were mixed by inversion for a dozen times. After decanting for an hour, 1 ml of aqueous phase was transferred in 2 ml eppendorf tubes containing 200 mg sodium sulfate dehydrated and vortexed for 2 sec. Dehydrated phase containing chloroform was transferred into vials and measured by gas chromatography using an injector 7683B Series (Agilent). A range of standard solutions, from 0 to 2 g/L, has been analyzed in parallel to the samples. Chromatograms were analyzed with the software HP1100.

Acetaldehyde assay was done inducing its oxidation to acetic acid in presence of the enzyme aldehyde dehydrogenase and NAD^+. The reaction causes NADH formation which is stoichiometric to the quantity of acetaldehyde consumed and then present in the sample. NADH is quantified spectrophotometrically at 340 nm. Briefly: in a spectrophotometric cuvette were added subsequently 1,5 ml of premix (an aqueous solution containing 0,1% NAD (cat. no. 43410, Fluka) and 6,66% pyrophosphate buffer (cat. no. 32431, Aldrich)) and 50 µl of sample (or water in the case of the blank). After vortexing optical density at 340 nm were measured (A_1). Ten µl of aldehyde dehydrogenase (cat. no. A6338, Sigma) were added to start the reaction, cuvette was immediately sealed with Parafilm® and mixed by inversion. After few minutes optical density was measured again (A_2). Acetaldehyde concentration was calculated following the formula:

$$[\text{Acetaldehyde}] = a\,[217{,}9*(\Delta_{A\ \text{sample}} - \Delta_{A\ \text{blank}})] + b$$

where :

$\Delta_A = A_2 - A_1$

a and b calculated from calibration results.

Samples to quantify volatiles compounds were prepared for GC/MS analysis by adding 5 deuterated standards (*d5*-ethylbutanoate, *d5*-ethylhexanoate, *d5*-ethyloctanoate, *d5*-ethyldecanoate, *d7*-butanoic acid, *d4*-2-phenylethanol) to 5 ml sample and extracting twice with 1 ml of dichloromethane. The resulting emulsion, after each extraction, was dispersed by centrifugation (3000g for 5 min). The organic extracts were dried over anhydrous sodium sulphate. Volatiles compounds were analysed with an Agilent 6890N gas chromatograph equipped with a PAL autosampler coupled to a mass spectrometer (electronic impact 70 eV). The gas chromatograph was fitted with a Phenomenex fused silica capillary column ZB-WAX column (30m * 0.25mm I.D. * 0.25 µm Film thickness, Zebron Phenomenex). The carrier gas was helium, flow rate 2.0 ml/min in constant flow mode. The oven temperature was started at 40°C, held at this temperature for 3 min, then increased to 220°C at 4°C/min and held at this temperature for 20 min. The injector was set to 250°C and used in splitless mode (10:1, for 30 sec). The mass spectrometer quadrupole temperature was set at 106°C, the source was set at 230°C and the transfer line wax held at 250°C. Positive ion lectron impact spectra at 70 eV were recorded in selective ion monitoring (SIM). Agilent MSD Chemstation software was used for instrument control and data processing.

4.2 Results:

4.2.1 Evaluation of the role of FRE genes in wine fermentation/velum ageing adaptation

FRE genes represent an interesting family of genes presenting several variations suggesting that they might play a significant role. These genes are involved in the transport of iron and encode ferric reductases. They reduce the chelated iron so that it can be transported inside the cell by the complex Fet3p/Ftr1p. In an aerobic environment the iron is quickly oxidized to the ferric form, poorly soluble in water, that cannot be assimilated by the cells. Microorganisms synthesize and secrete siderophores that can bind iron outside the cell and be selectively recognized by ferric reductases. This complex solubilizes the iron in the extracellular environment and is selectively recognized by a specific transport mechanism that takes it up inside the cell (Byers and Arceneaux, 1998). There are 9 *FRE* genes (Fig. 1): *FRE1* and *FRE2* are ferric and cupric reductases (Georgatsou et al., 1997), *FRE7* is a cupric-reductase, *FRE3*, *FRE4*, *FRE5* and *FRE6* are ferric-reductases while the transcription of *FRE8* and *YGL160W* is not modified by iron or copper (Georgatsou and

Alexandraki, 1999). Fre3p and Fre4p bind ferric iron linked to different siderophores (Yun et al., 2001), while Fre5p and Fre6p are localized respectively in mitochondria and vacuole (Sickmann et al., 2003; Huh et al., 2003). Under low iron conditions Aft1p activate *FRE* genes except *FRE7*, *FRE8* and *YGL160W*. The transcription of *FRE1-6* genes is induced under low iron conditions, and *FRE2* and *FRE3* are the more induced (Dancis et al., 1990; Anderson et al., 1992). *FRE1* is induced even by Aft2p and, like the other cupric reductase *FRE7*, by Mac1p under low copper conditions. *FRE2*, even if considered a ferric/cupric reductase, seems to be not regulated by Mac1p (Georgatsou and Alexandraki, 1999).

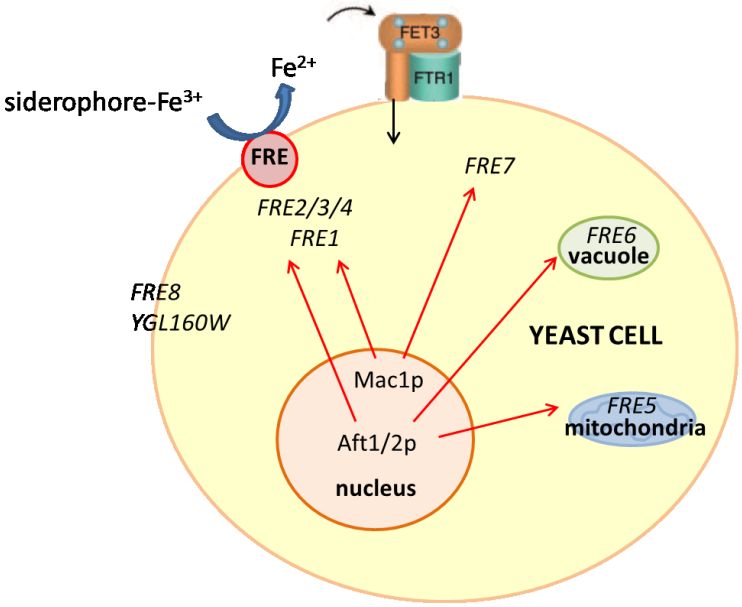


Figure 1: Schematic representation of iron uptake and *FRE* genes localization inside yeast cell.

The alignment of sequence reads of *FRE2* flor strains to the reference genome of S288c with BWA software revealed a complex pattern of recombination at these loci.

No reads were mapped in a short region inside the *FRE2* ORF, for strains GUF54-A1 and 7.7 (Fig. 2A) and for reads that were mapped on the sides of this region, their mate pair where mapped on another chromosome (materialized with a purple color) at *FRE3* locus (whereas the read should be mapped at a 300bp maximum distance) indicating a translocation. Symmetrically, in the *FRE3* region (Fig. 2B), a small region pointed by the paired read of Chromosome XI displayed a sudden increase of the number of mapped reads indicating a higher number of copies of that DNA fragment in the genome. Besides this region another small region of *FRE3* displays inversely the same phenomenon for strains

TS12, 2D, 7.7 and F25. These data suggest a recombination of *FRE2* and *FRE3* genes. In order to confirm this rearrangement, we resequenced *FRE2* and *FRE3* genes with the classical Sanger method and we confirmed that the ferric reductase of *FRE3* is recombined with *FRE2* and vice versa. In some strains this recombination is homozygote (GUF54-A1 and 7-7), in other it is heterozygote (TS12-A7, 2D and F25), and 2 strains have the wild type allele of *FRE2* (P3-D5 and TA12-2). Thus in these strains a double exchange occurred between *FRE2* and *FRE3* genes.

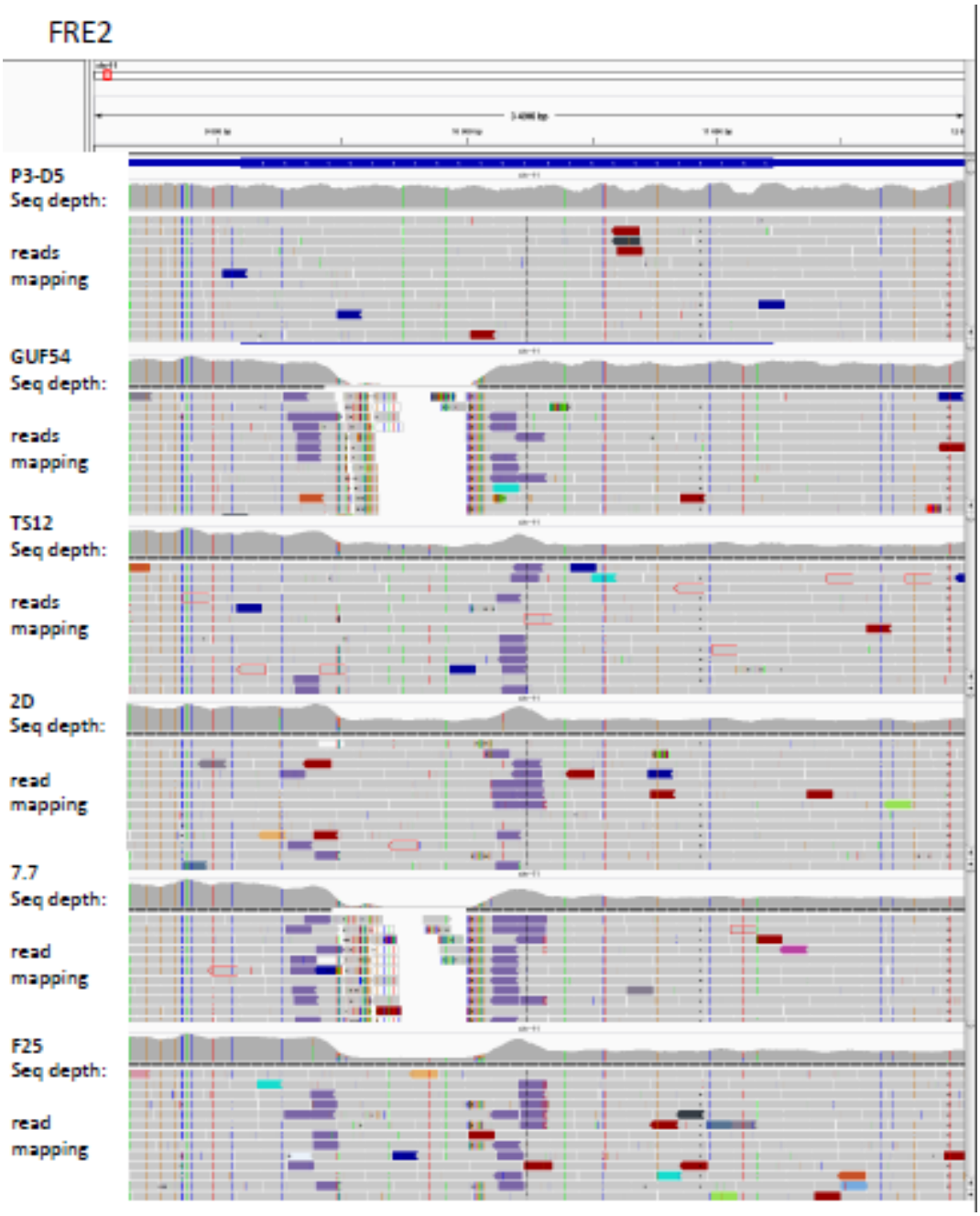


Figure 2A: Visualization of the result of read mapping in *FRE2* region obtained with BWA on IGV (Robinson et al., 2011; Thorvaldsdóttir, Robinson, & Mesirov, 2013). For each strain, the sequencing depth is drawn along the selected region on the first lane, read mapping is presented in the lane below. SNPs are indicated by a color bar, pairs of reads correctly mapped are in grey. Reads with discordant paired end mapped on chromosome XV are in purple.

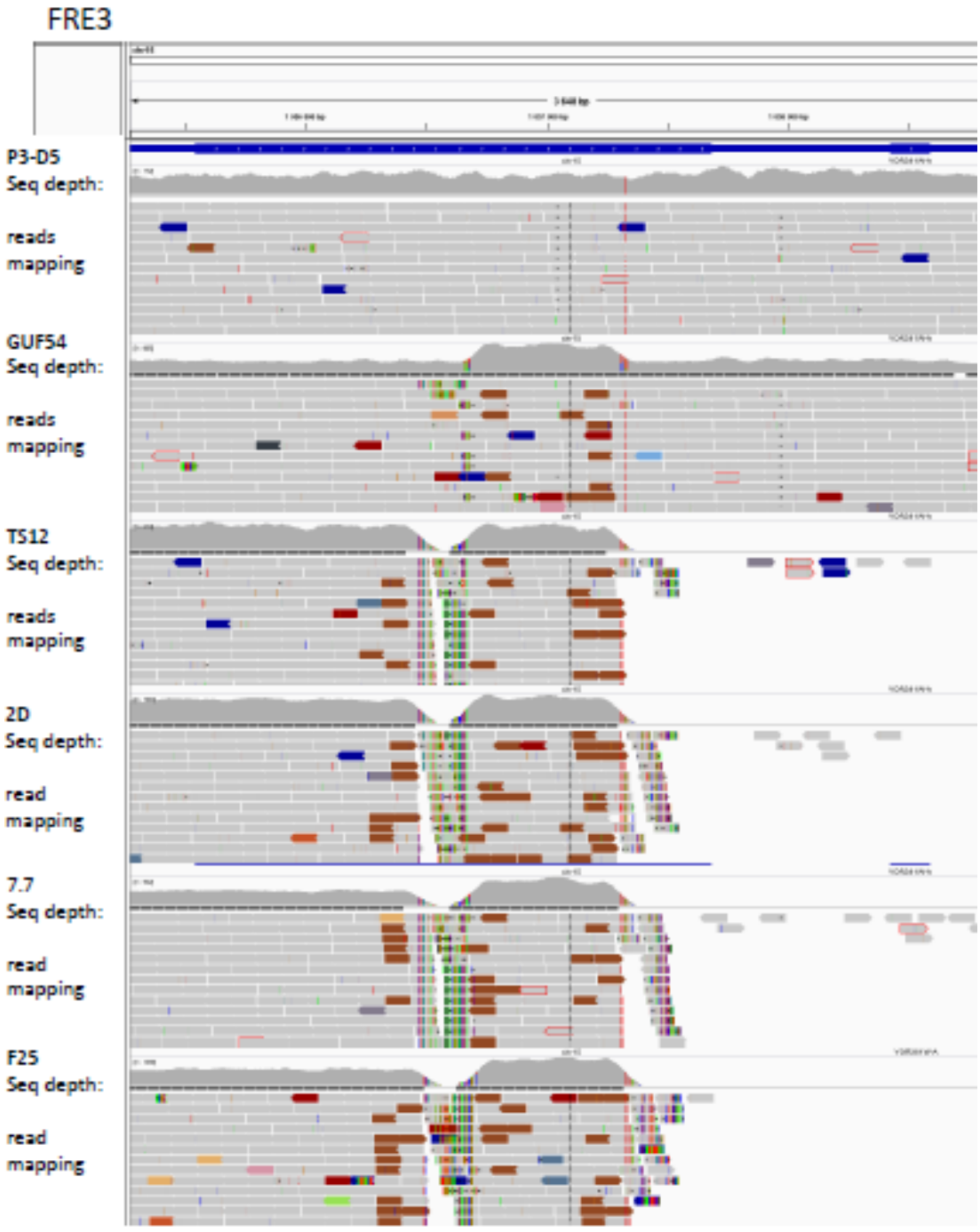


Figure 2B: Visualization of the result of read mapping in *FRE3* region obtained with BWA on IGV (Robinson et al., 2011; Thorvaldsdóttir et al., 2013) as for *FRE2*. Reads with discordant paired end mapped on chromosome XI are in brown.

Furthermore, strains TS12, 2D, F25 and 7.7 present another recombination with *FRE2*: several reads close to the end of the ORF of *FRE3* are mapped in *FRE2* or are not mapped at all. This creates a gap corresponding to the 7 following genes on the chromosome XV that appeared as deleted:

- *FIT2* and *FIT3*: encode for two GPI-anchored mannoproteins involved in the retention of the complex siderophor-iron in the cell wall;
- *FRE5*: one of the ferric reductases with mitochondrial localization;
- *PHR1*: a DNA photolyase involved in photoreactivation;
- *YOR387c*: a putative protein, regulated by Aft1p and highly inducible in low zinc conditions;
- *YOR381W-A* and *YOR385W*: two non-essential genes of unknown function.

It is interesting to note that the deletion of *FIT* genes, individual or in combination, leads to defects in the uptake of iron bound to some siderophores, without diminishing the uptake of ferric ion salts and of the iron bound to other siderophores and that results in an increased expression of Aft1p target genes among which *FRE* genes (Protchenko et al., 2001).

Recombination between *FRE3* and the terminal part of *FRE2* was confirmed by PCR (Fig. 3) using a primer FRE3Fw and a primer FRE2Rv. The pair end of 1/3 of reads matched on *FRE3* match with *FRE2*, whereas for the other 2/3 all mapped reads have their paired end mapped 5' to the read: we are unable to know the 3' end of the gene. It could be interesting to detect to what *FRE3* is recombined with. This would enable us to understand if NAD and FAD binding domains of *FRE3*, responsible of oxidation-reduction process and of the oxidoreductase activity (**string**-db.org/) have been replaced by those of *FRE2* and, consequently, understand if these recombined alleles of *FRE3* are functional. For this longer reads are required in order to obtain better assemblies, or another sequencing strategy such as can be achieved with mate paired reads.

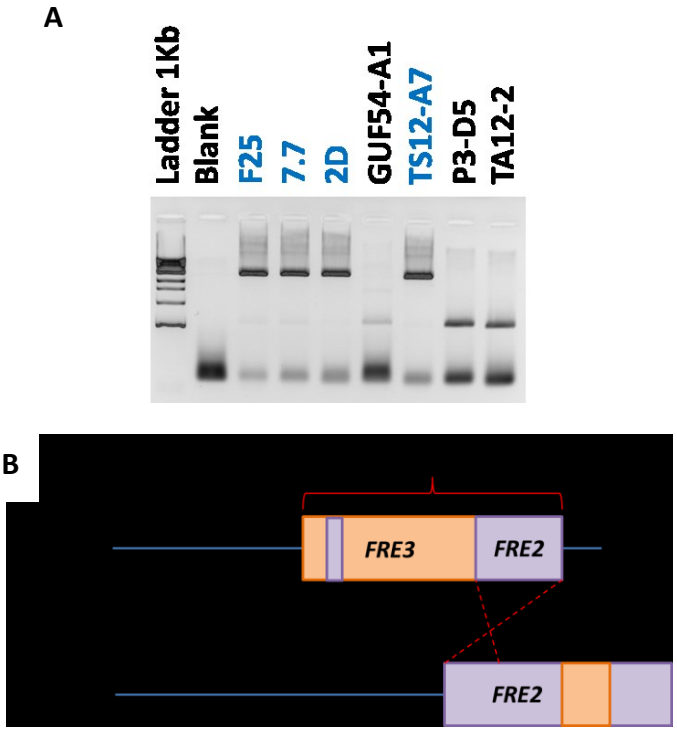


Figure 3: A) Amplification of *FRE3*/*FRE2* chimeric gene. In blue are represented strains that present recombination. B) Scheme of possible *FRE3*/*FRE2* recombination.

Interestingly, another *FRE* gene, *FRE6*, encoding a ferric-reductase analogous to *FRE2* and localized in the vacuole (Huh et al., 2003), presents also a high number of mutations. In Fig. 4 is represented the phylogeny of *FRE6*. In the longest branch is present the allele of *Saccharomyces paradoxus*, flor strain allele is located in the long branch in the top of the tree, wine strain alleles are located on another branch from the opposite side of flor allele. This specific phylogeny is also confirmed by a high neutrality index (3.0) and a significant Mc Donald Kreitman test indicating a possible balancing selection at this locus.

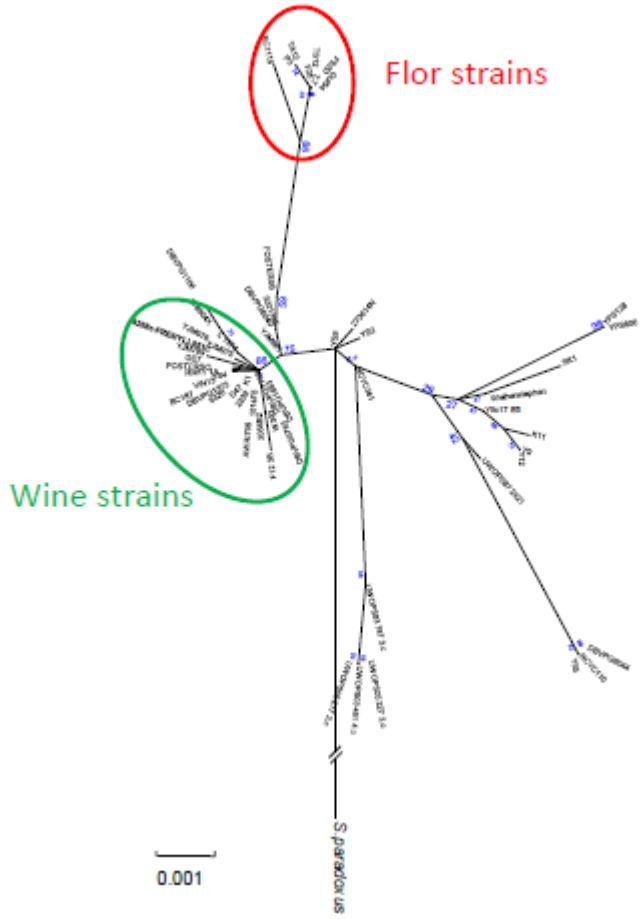


Figure 4: Neighbor joining tree presenting the phylogeny of *FRE6*. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) using 59 nucleotide sequences from the available genome sequence.

Iron is an important constituent of the electron transport chain, therefore its presence is essential for yeasts that live in an aerobic environment like flor strains. Furthermore, during the diauxic-shift there is an increase in iron uptake mediated by high affinity transport systems (Haurie et al., 2003). An additional consideration is that the cell responds to iron deficiency and to the absence of *FIT* genes by up-regulating the systems of iron uptake. In this context, we can wonder if the mutations in *FRE2* and *FRE6* can lead to a better absorption of iron, even for strains for which *FRE5* is missing or *FRE3* apparently truncated. To answer this question we tried to construct wine and flor *FRE2*, *FRE3* and *FRE2-3* allelic replacement mutants. We succeeded in constructing wine *fre2Δ*, *fre3Δ*, the double deleted mutant *fre2,3Δ* and the flor deleted mutant *fre2Δ* but it was not possible obtain the flor *fre3Δ* and therefore the flor *fre2,3Δ*. We were not able to delete *FRE3* in all

haploid flor strains previously created (P3-D5, GUF54-A1 and 2D), and neither to amplify this gene from flor strains, whereas we could obtain amplification in wine strain in which we could delete *FRE2*. The lack of amplification from the strain 2D was not surprising because of the mismatch after the ferric reductase domain, while in P3-D5 and in GUF54-A1 there is no apparent reason for that. More work has to be done in order to evaluate the surrounding of *FRE3* in flor yeasts.

However another possibility can be the opposite, the lack of selective pressure at these loci leading to the accumulation of mutation and loss of function of these alleles. Indeed, the phenotypic effect might be related to the gene *MCH2* which codes for a protein with similarities with a carboxylic acid transporter, but without known activity in *S. cerevisiae* (Makuc et al., 2001) or the gene *YKL222C*, coding for a protein of unknown function, which are adjacent to *FRE2*. These genes are found in several copies as it can be noticed from the Fig. 2. In agreement with this, a second translocation is found between *FRE2* and *MCH2* in the genome of F25, between chromosome XI and chromosome V leading to a truncated *HXT13*. In this triploid strain which was sequenced at a depth of 700X, we could see the number of reads dropping at 250, inside *FRE2*, and then increasing again to 1500 copies after *FRE2*, 2400 at *MCH2* and 3300 copies for *YKL222c*. These genes were the sole genes that were found amplified in flor strains in a CGH array study performed before (Legras et al., submitted).

4.2.2 Evaluation of the role of PIK1 gene in adaptation to velum ageing

Among the divergent genes not involved in metal transport, the gene *PIK1*, which encodes a phosphatidylinositol 4-kinase, is found in a highly polymorphic region: 11 mutations are found in its coding region which leads to four amino acid substitution Pro330 Ser, Asn338 Ser, Ser382 Phe and Gly596 Asp but in addition its promoter region is remarkably divergent for flor yeasts (Fig. 5). A Mc Donald Kreitman test (McDonald and Kreitman, 1991) calculated for the promoter region, using *PIK1* coding sequence as a reference, indicates a non-neutral evolution of the promoter region (NI = 7.6; p-value <0.001).

Interestingly, the phylogeny of *LYP1* (500bp upstream *PIK1*) is similar to the global phylogeny however flor strain *PIK1* promoting region and *PIK1* are much closer from Asian strains, suggesting an introgression from a more distant origin as well (Fig. 6).

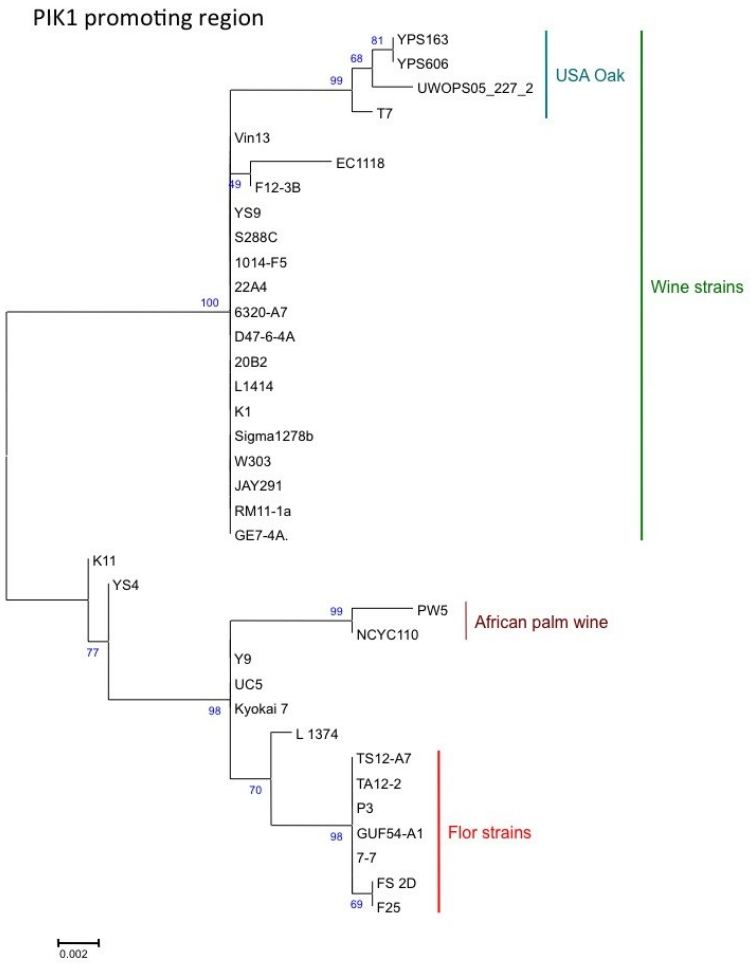


Figure 5: Neighbor joining tree presenting the phylogeny of *PIK1* promoter. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) using 36 nucleotide sequences from the available genome sequence.

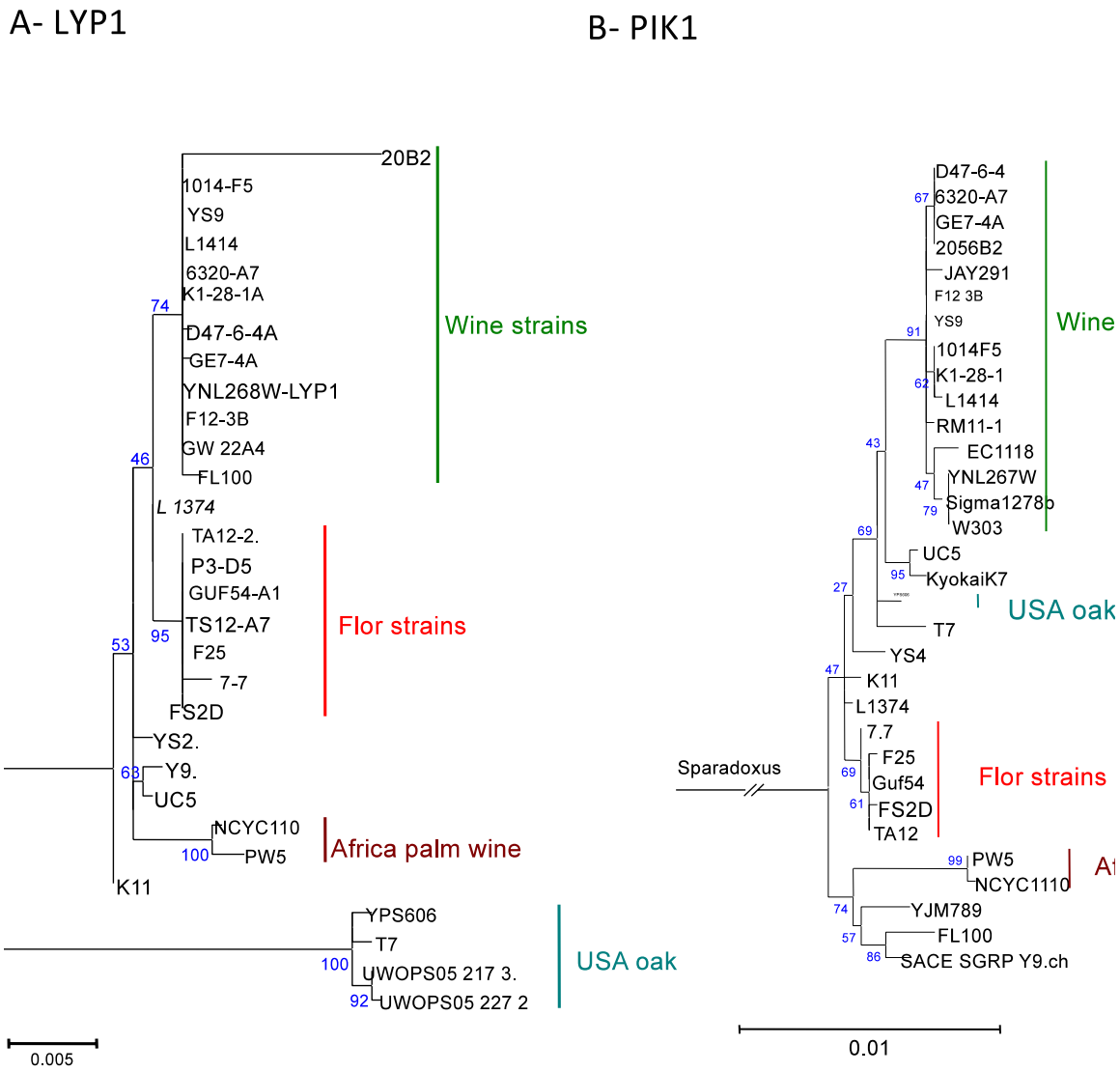


Figure 6: Neighbor joining tree presenting the phylogeny of *LYP1* (A) and *PIK1* (B). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) using 29 and 32 nucleotide sequences from the available genome sequence for *LYP1* and *PIK1* respectively.

PIK1 encodes a phosphatidylinositol 4-kinase (PtdIns 4-kinase) that catalyzes the formation of phosphatidylinositol 4-phosphate, precursor of phosphatidylinositol 4,5-biphosphate (Flanagan et al., 1993; Garcia-Bustos et al., 1994). *PIK1* contains in its promoter a UAS_{INO} element and, in the presence of inositol, is negatively regulated by Ino2p, one of the three transcription factors of the inositol regulon which controls expression of many phospholipid biosynthetic genes (Wimalarathna et al., 2011; Greenberg and Lopes, 1996). The same regulation is observed for *INO1*, coding for a key enzyme of

inositol biosynthesis, which is up-regulated in mat cultures (Ambroziak and Henry, 1994; Reynolds, 2006). Furthermore, one of the components of the inositol regulon, Opi1, controls biofilm formation by regulating the expression of Flo11 (Reynolds, 2006) and velum production is affected by inositol concentration (Zara et al., 2012). Pik1 has different functions inside the cell: it regulates secretion in the Golgi, has a role in endocytosis, in the vacuole and Golgi membrane dynamics and can control cytokinesis through the actin cytoskeleton (Audhya et al., 2000; Hama et al., 1999). Another function of *PIK1* is to regulate the activity of three different Mitogen-activated protein kinase (MAPK) in yeast: mating, filamentous growth (FG) and High Osmolarity Glycerol (HOG) pathways (Cappell and Dohlman, 2011). These MAP kinase pathways respond to different external stimuli: the first pathway is activated by the mating pheromones and induces the mating program; the second pathway responds to nutrient deprivation and induces filamentous growth, increased adherence and invasion; the third pathway is activated by osmotic stress, activates the MAPK Hog1 and induces glycerol production to counterbalance osmotic pressure. Pik1 is required for full activation of the MAP kinases Fus3 and Hog1 responsible of the activation of mating program and high osmolarity glycerol response and represses activation of Kss1 which induces filamentous growth (Cappell and Dohlamn, 2011). A last unsuspected aspect is that three genes involved in divalent metal transport: *SMF1*, *ALR2* and *ZRT3*, among the 142 genes interacting with *PIK1* lead to synthetic growth defects (Demmel et al., 2008). As *PIK1* contributes to filamentous growth we hypothesized that mutations in *PIK1* promoter region of flor strains could be involved in the aptitude of these strains to produce biofilm.

In order to analyze a possible role of these mutations in biofilm formation, we decided to perform the allelic replacement of a fragment of *PIK1*-flor promoter in wine strains that contained most of the mutations and of its corresponding fragment *PIK1*-wine promoter in flor strains.

Phosphatidylinositol 4-kinases are essential genes and deleted mutants are inviable (Flanagan et al., 1993; Yoshida et al., 1994). We deleted, then, the most mutated part of the promoter. The site where we inserted the KanMX cassette in the strain usable to carry out the allelic replacement, that we have not yet completed, and the portion deleted were located next to the end of *LYP1*'s ORF, a gene that encodes a lysine permease (Fig. 7).

Analyzing the portion of promoter that we wanted to exchange (http://www.yeastract.com/) we identified different putative transcription factor binding sites (Fig. 7). Among them Tec1, a transcriptional activator of most filamentation gene promoters that works in association with Ste12 (Chou et al., 2006), is located in the flor promoter whereas Nrg1, a transcriptional repressor of filamentation, invasive growth and biofilm formation (Kuchin et al., 2002), is found in the wine allele of the promoter. Furthermore, the production of phosphatidylinositols has been found recently to be required for *FLO11* expression (Guillas et al., 2013).

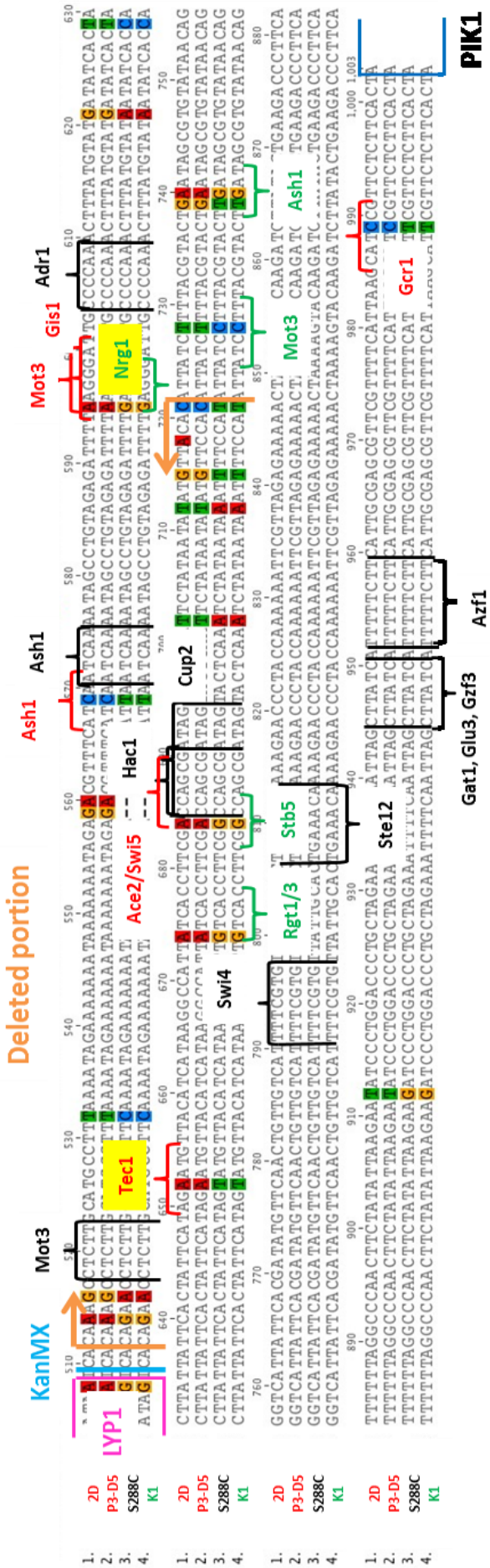


Figure 7: *PIK1* promoter sequence of the strains 2D, P3-D5, S288C and K1.

A protein structure prediction made using the RaptorX web server (Kallberg et al., 2012) indicates the presence of slight differences in the folding of the flor and wine Pik1 proteins (Fig. 8). The secondary structure prediction revealed differences in the distribution of secondary structure classes: 46% helix, 4% beta-sheet and 48% loop for flor Pik1 while 46% helix, 5% beta-sheet and 47% loop for wine Pik1. Some variations concerning solvent accessibility in the medium state, which comprises cutoff values between 10% and 42%, were also predicted: 61% for flor Pik1 and 60% for wine Pik1. Prediction of binding sites, also carried out using RaptorX, revealed binding residues: some of them were shared by both flor and wine proteins (I794, K796, E804, M830, V842, E843, T844, I845, A848, M925, I935, D936,) while 4 binding residues were exclusively detected in flor Pik1 (L509, E513, V515, P516) but none of them corresponds to flor amino acidic substitutions. Another difference was in the order of ligands likelihood of binding the query protein: for flor Pik1 protein the order was 093, 090, AJZ, X6K, 3MA while for wine Pik1 was 090, 3MA, AJZ, X6K, 093. Ligand 093 is N-(5-(4-chloro-3-(2-hydroxy-ethylsulfamoyl)-phenylthiazole-2-YL)-acetamide and ligand 090 is N-(2,3-dihydro-7,8-dimethoxyimidazo[1,2-C] quinazolin-5-YL) nicotinamide, two small not well characterized molecules that both bind the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma isoform and the phosphatidylinositol 3-kinase catalytic subunit.

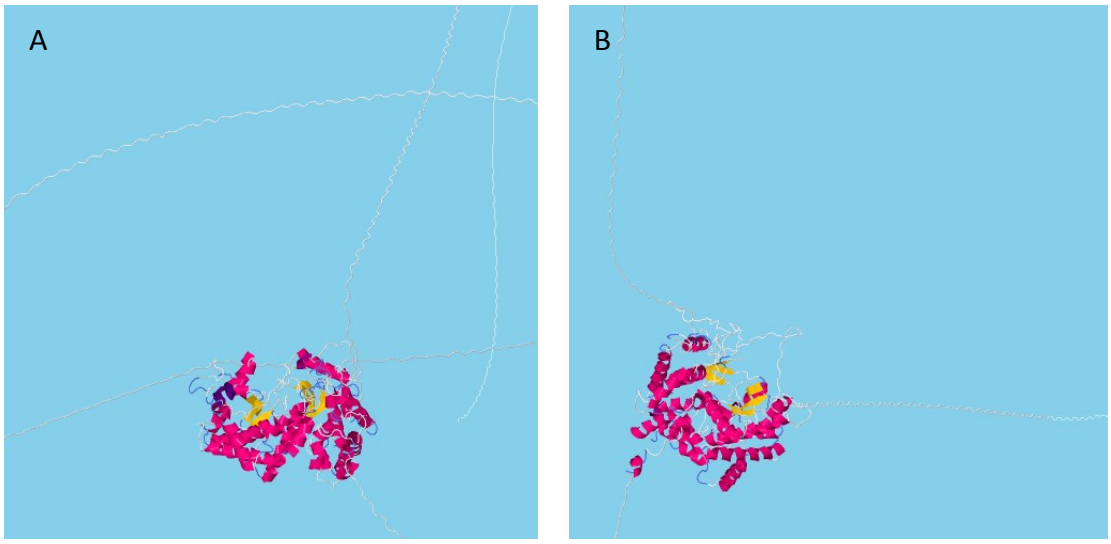


Figure 8: Tertiary structure models predicted using RaptorX. A: Pik1 flor model. B: Pik1 wine model. The relative quality of predicted models: pvalue 1.58e-09 for flor Pik1 and 1.07e-09 for wine Pik1.

4.2.3 Evaluation of the role of PDC1 gene in adaptation to velum ageing

The major pyruvate decarboxylase gene *PDC1* is another gene where we could observe a recombination. This *PDC* class of genes encode for pyruvate decarboxylase, enzyme that catalyzes the decarboxylation of pyruvate to acetaldehyde. Their synthesis is induced by high concentration of glucose at the transcriptional level (Butler and McConnell, 1988; Kellermann and Hollenberg, 1988; Schmitt et al., 1983). This homotetrameric enzyme uses as cofactors thiamine diphosphate and magnesium ions and is activated allosterically by pyruvate and inorganic phosphate (König, 1998; Boiteaux and Hess, 1970). There are 4 *PDC* genes: *PDC1*, *PDC5* and *PDC6* encoding pyruvate decarboxylases, while *PDC2* has a regulatory activity (Schmitt and Zimmermann, 1982; Wright et al., 1989). *PDC1* is the main isozyme, *PDC5* is expressed only in absence of *PDC1* or under thiamine limitation (Schmitt and Zimmermann, 1982; Seeboth et al., 1990; Muller et al., 1999). Both are expressed during glucose fermentation, are under Pdc1p autoregulation and seem to be repressed during growth on ethanol (Eberhardt et al., 1999; Liesen et al., 1996). Their expression is activated by Pdc2p (Mojzita et al., 2006). Deletion of both *PDC1* and *PDC5* results in the complete loss of *PDC* activity, in failure to ferment glucose and to grow normally on a glucose medium. *PDC6*, instead, is not expressed during glucose fermentation but seems to be activated during growth on non-fermentable carbon source (Hohmann, 1991). *PDC* genes have also secondary functions: they are involved in the catabolism of some amino acids like isoleucine, phenylalanine, tryptophan and valine through the decarboxylation of α-ketoacids (Dickinson et al., 1997; Dickinson et al., 2003) and in the detoxification of acetaldehyde. In fact, *PDC* genes can promote the reaction of the acetaldehyde with a molecule of acetaldehyde activated by the binding with thiamine diphosphate to obtain acetoin, which is then metabolized to 2,3-butanediol. The proportion of acetoin produced increases with increasing concentrations of acetaldehyde (Juni, 1961) and Sherry wines contain a very high level of acetoin (Crowell and Guymond, 1963). Indeed, even pyruvate, in presence of pyruvate decarboxylase, can interact with active acetaldehyde forming acetoin (Juni, 1952; Romano and Suzzi, 1996). This unusual role of the pyruvate decarboxylase to catalyze carbon-carbon bond formation seems due to its homology with acetolactate synthase, which catalyzes this step in valine and isoleucine biosynthesis (Green, 1989).

As observed for *FRE2* and *FRE3*, the mapping of the sequence reads showed a marked drop inside *PDC1* gene, and pair ends mapping on *PDC5* gene indicating a recombination between *PDC1* and *PDC5* of all flor stains (Fig. 9). This is confirmed by the sequence of this gene obtained in the final genome assembly for several strains. This recombination corresponds to the central thiamine pyrophosphate binding domain of *PDC1* and *PDC5* genes. *PDC5* is however normal in all flor strains. In order to detect any phenotypic variations connected to these allelic variation, and detect a potential advantage in fermentation or flor ageing environment, such as in a detoxification role, we chose to introduce each *PDC1* allele in a strain without any *PDC* activity, CEN.PK *pdc1,5,6*Δ, and to monitor the formation of acetaldehyde, acetoin and 2,3-butanediol produced by the different enzymatic activities of *PDC1* both during fermentation and aerobic culture.

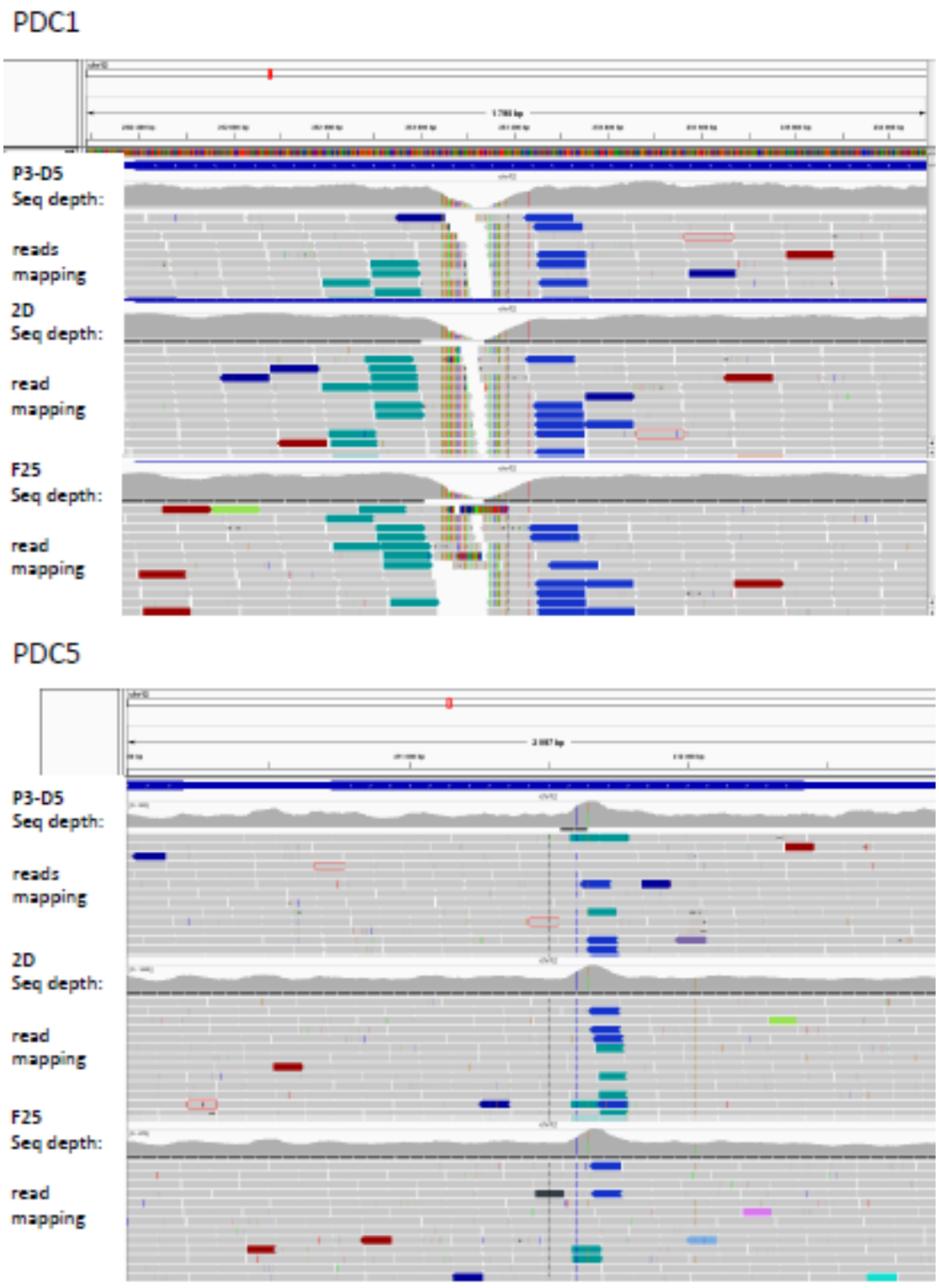


Figure 9: Visualization of the result of read mapping in *PDC1* and *PDC5* regions obtained with BWA on IGV (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). Reads with discordant paired end mapped on chromosome XII are in blue.

In order to compare the two alleles of *PDC1*, we chose to compare them using a strain without any PDC activity. As CEN.PK *pdc1,5,6*Δ is only able to use glycerol or ethanol as

a carbon source media but not glucose, the introduction of *PDC1* has been performed at the *PDC1* locus, without using an antibiotic cassette and transformants were selected on YEPD.

The two mutant strains were first characterized for their growth and metabolic production during aerobic cultivation on Flor Medium 8% ethanol + 10X vitamins in order to use a condition similar to biological ageing (Fig. 10). As strains were auxotrophic (MATa pdc5(-6,-2)::loxP; pdc6(-6,-2)::loxP ura3-52; YLR044C::YLR044C-2D) 0.01 mg/ml uracil were added.

No differences were observed in the growth of allelic replacement mutants: curves were perfectly overlapped, and concentration of acetaldehyde, acetoin and 2,3-butanediol produced during aerobic growth were too low to be quantified.

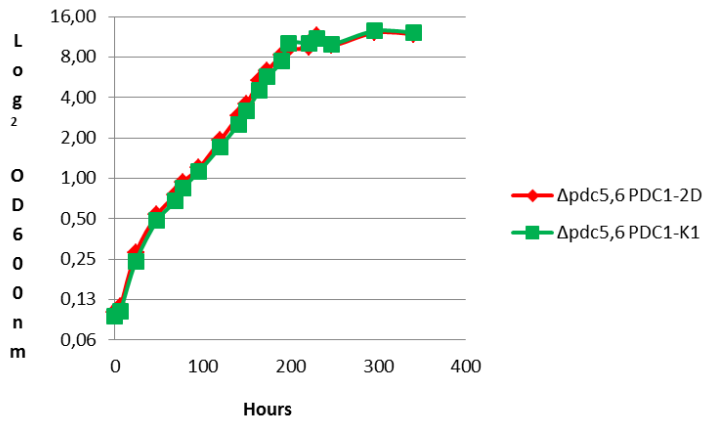


Figure 10: Aerobic growth of mutant strains carrying *PDC1* flor allele (red) or *PDC1* wine allele (green) on flor medium 8% ethanol + 10X vitamins.

An alternative could have been to evaluate metabolic production during fermentation. We used a modified version of the synthetic must MS 300 described by Bely and colleagues (1990) and monitored fermentations for 100 hours. Fermentation kinetics of the two mutants overlapped showing that flor *PDC1* recombination did not change fermentation rate (Fig. 11). Also the quantification of metabolites produced by pyruvate decarboxylase activity of Pdc1 revealed no significant differences between flor and wine Pdc1: acetoin, 2,3-butanediol and acetaldehyde were produced in the same quantity in the two mutant strains carrying flor or wine allele (Fig. 12).

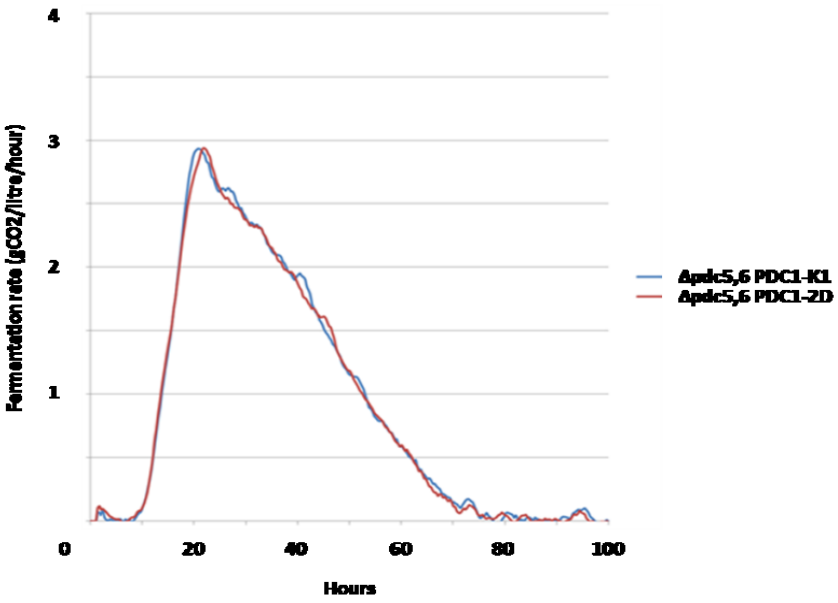
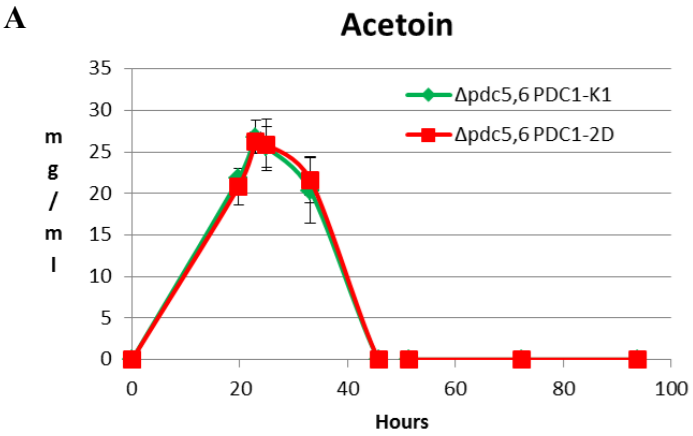


Figure 11: Fermentation kinetics of *PDC1* mutants. In blue is represented strain carrying wine allele, in red strain with flor allele. Curves are representative of 3 independent experiments.



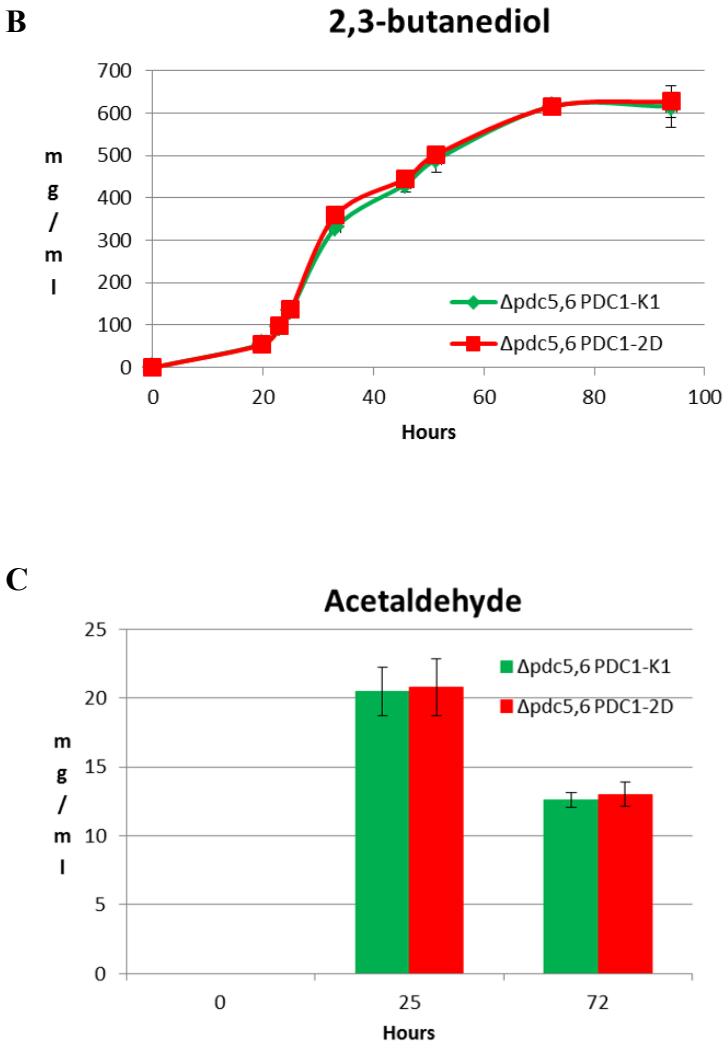


Figure 12: Acetoin (A), 2,3-butanediol (B) and acetaldehyde (C) production in the mutant strains carrying *PDC1* flor allele (red) or *PDC1* wine allele (green). Values are the average of three independent experiments.

Another possible differential function of flor *PDC1* recombined allele was that it could lead to the production of 2-phenylethanol or butanol, two secondary alcohol that may have an impact in *quorum sensing* (Wuster & Babu, 2010). In order to check this hypothesis we measured the production of volatile aroma compounds at the end of fermentation. We did not find differences in the production of 2-phenylethanol or butanol, but we detected a significant increase in the production of some ethyl esters and fatty acids in the mutant strain carrying *PDC1* wine allele (table 3). Thus, under our conditions *PDC1* wine allele displayed a better pyruvate decarboxylase activity than flor allele, that we did not detect as well neither from fermentation kinetic nor from the production of ethanol. The recent comparison of Pdc1 and Pdc5 enzymatic properties revealed that the two enzymes have

close activities, but *PDC5* presents a higher Vmax but a slightly lower affinity for pyruvate (Agarwal, Uppada, & Noronha, 2013). These authors could not differentiate as well growth obtained with strains carrying only one allele on YEPD medium.

Other results obtained with several flor and wine strains revealed that flor strains present a global higher *PDC* activity (Nidelet et al., unpublished results). This suggests that either the choice of a CEN.PK pdc$^-$ strain for comparing the two alleles was not optimal for the comparison to flor strain: but maybe other factors such as thiamine availability should be taken into account as thiamine limitation bring to *PDC5* expression (Muller et al., 1999) for detecting different activity of these enzymes. Alternatively, the global *PDC* activity observed for flor yeast may have another origin.

Table 3: Volatile compounds production in the mutant strains carrying *PDC1* flor allele (Δpdc5,6 *PDC1*-2D) or *PDC1* wine allele (Δpdc5,6 *PDC1*-K1).

Volatile compounds **Strains**	**Δpdc5,6 *PDC1*-2D**	**Δpdc5,6 *PDC1*-K1**
Ethyl butanoate	0.36±0.01^b	0.38±0.01^a
Ethyl hexanoate	0.49±0.04^b	0.56±0.03^a
2-phenylethyl acetate	1.02±0.07^b	1.18±0.07^a
3-methylbutanoic acid	0.47±0.02^b	0.50±0.02^a
Hexanoic acid	3.09±0.13^b	3.29±0.05^a
Octanoic acid	2.81±0.20^b	3.39±0.13^a
Decanoic acid	1.06±0.18^b	1.61±0.12^a

4.2.4 Evaluation of the role of ZRT1 gene in wine fermentation/velum ageing adaptation

ZRT1, coding for a high affinity zinc transporter, is the gene that presents the highest divergence between wine strains and flor strains. As described in the chapter 3 we examined the role of flor and wine alleles in relation to a possible antioxidant function, but the two alleles did not exhibit differences both under oxidative stress and zinc-limiting conditions.

We decided, then, to carry out a Phenotype Microarray analysis in order to detect phenotypic variations among different *ZRT1* mutants. This technique, with the use of 96-well plates carrying 96 different conditions, allows measuring a big amount of cellular

phenotypes at the same time. PM technology consists in a colorimetric reaction that exploits cellular respiration: when cell grow normally there is a flow of electrons that goes to the tetrazolium dye. The reduction of the dye caused the formation of a purple color (Bochner et al., 2001). With this technique we tested *ZRT1* mutants and wild-type for 90 different carbon sources, 95 nitrogen sources, osmolarity and pH gradient (data not shown). In none of these conditions, it was possible to find significant and reproducible differences between flor and wine *ZRT1* alleles.

4.3 Conclusions

Flor strains present many genetic peculiarities that remain to be evaluated. The difficulties that we observed for *FRE2* and *FRE3* genes are partly related to an insufficient knowledge on the assembly made in these regions because of multiple translocations. But it is possible that even if these translocations create novel alleles, these are maybe not the cause of the selection of this event, but instead the increase in copy number at *MCH2* or *YKL222C* loci. However, an interesting point arises from the analysis of these strains: the French strain P3-D5 as well as the Hungarian strain TA12-2 present less translocations on *FRE2-3* genes when compared with Spanish and Italian strains, do not have a mutated version of *ICR1*, the long intergenic non-coding RNA regulating *FLO11* expression, and sporulate easily, so that these strains appear to be closer from the ancestral type of flor strain and thus less evolved than these Mediterranean strains.

PIK1, encoding a phosphatidylinositol 4-kinase, among the various roles played regulates also the filamentous growth pathway in yeast. The analysis of deleted promoter fragment revealed several transcription factor binding sites: among them Nrg1 in the wine portion and Tec1 in the flor portion. The exchange of this mutated fragment of promoter will provide informations about a potential role of this gene in the regulation of biofilm formation. Furthermore, a protein structure prediction displayed a different folding of flor and wine Pik1 proteins and differences in binding residues suggesting that also mutations in the ORF could display a role in the regulation of biofilm production.

As *FRE2-3* genes also *PDC1* presents recombination with a gene that belongs to the same family. Since we were not able to detect clear differences in the enzymatic properties of

PDC1 mutants, we have to exclude a role of the flor allele in the detoxification of acetaldehyde. In fact, as it is not known if *PDC1* and *PDC5* display different activities, it is possible that this translocation may result in unchanged enzymatic activity of Pdc1. Another possibility was that Pdc1 could lead to the production of secondary alcohols, that may have an impact in quorum sensing (Wuster & Babu, 2010). The quantification of aromatic compounds produced during fermentation did not confirm a potential role in 2-phenylethanol production and suggested a higher *PDC* activity for the wine allele. More work is necessary to understand if under optimal conditions the higher pyruvate decarboxylase activity of flor strains detected in other studies (Nidelet et al., unpublished results) is due to *PDC1*/*PDC5* recombination in flor strains.

5. DISCUSSION

Wine and flor yeast are two strains living in two contrasted environment. Different authors have illustrated through different studies of both wine and flor strains how mutations are an important mechanism used to adapt to an hostile environment, (Pérez-Ortín et al., 2002; Fidalgo et al., 2006; Guillaume et al., 2007). However until now only *FLO11* was found as an example of "domestication-related gene" (Meyer & Purugganan, 2013) for flor yeast, whereas many more variations could be expected from the huge differences in their living style. In this PhD thesis we have studied the genetic specificities of flor strains in comparison to wine strains, in order to identify the genetic features that may explain the adaptation of these yeasts to their ecological niche. To this end, we have used a population genomic approach based on genome sequencing of 10 flor strains and 8 "classical" wine strains. Our aim was also to combine this comparative genomics approach to functional studies, in order to shed light on the potential impact of the identified variations for flor strains. To achieve this goal, we developed specific tools: a set of model flor and wine haploid strains and a synthetic flor medium.

The widely used laboratory strains share deficient genes (Kobayashi et al., 1996; Gaisne et al. 1999; Ambroset et al., 2011). Furthermore, V5 and 59A, the haploid derivatives of commercial wine yeasts previously developed in the SPO laboratory both derive from a cross between a flor and a wine strain. Although these strains remain good model to study most metabolic features of wine yeasts (fermentative performance, metabolite production), they are not the ideal model to be used for a thorough characterization of the differences between wine and flor strains, which was the aim of this study. The set of haploid strains generated in this study using the sequenced strains as parental strains enabled us to directly validate the impact of flor and wine-specific alleles. The haploid flor strains obtained were derived from strains of different origin, exhibiting also different ability to form biofilm: an Italian strain belonging to the "Mediterranean" subgroup and forming a thick velum, and a French strain belonging to the "Central Europe" group forming a thin velum which appears to be closer from the ancestral type of flor strain and hence less evolved than Spanish or Italian strains. The availability of these model haploid flor strains from different origin may therefore be interesting for studying the diversity of velum formation in the future.

Another important tool created was a synthetic flor medium mimicking the wine at the end of fermentation. We thought that the media usually used for flor strains (YNB + ethanol) has an excess of vitamins and a composition too far from wine characteristics at the end of

fermentation, so that we could miss some specific phenotypes associated with wine ageing. Indeed, a variable ability of Jura strains to produce a velum on YNB ethanol media has been previously observed (Legras et al., personal communication).

Our comparative genomic study first revealed the specific phylogeny of Flor yeast, a new group of strains that appear in addition to those previously described (Liti et al., 2009). This group is close but separated from Wine group. Inside this new cluster we could even distinguish strains from "Central Europe group" consisting of Hungarian and French strains and the "Mediterranean group" formed by Spanish and Italian strains. In addition, some strains display a mosaic genome derived from Flor and Wine strains: a Lebanese strain MJ73 and the commercial strains EC1118, QA23 and VIN13. In a former work, the analysis of microsatellite diversity suggested that the origin of flor yeast should not be in Sardinia, Jura or Hungary; instead, the topology of the tree indicated that Spain or Lebanon were closer from a possible origin (Legras et al., submitted). Our genomic data do not support this hypothesis. The separation between "Mediterranean" and "Central Europe" and the topology of the tree still suggests a common origin. We cannot state that this origin should be Mesopotamia as suggested before for wine yeast from Lebanese MJ73 genome sequence as this strain has a mosaic genome. This does not rule out a possible Mesopotamian based origin for the flor yeast group, and typical flor strain have been isolated recently from Georgian flor aged wines (Capece et al., 2013) and that should be compared to the strain sequenced here.

Several methods can be been used to detect footprints of selection in genomes. According to the evolutionary time frame investigated one can favor comparative species test or population based test (Oleksyk, Smith, & O'Brien, 2010). In our case, Wine and Flor yeast group represent two close groups and it is likely that their differentiation is recent as attested from the phylogeny. Five methods can be applied to such case, as reviewed by Oleksyk et al. (2010): 1-local reduction in genetic variation, 2- changes in the shape of the frequency distribution of genetic variation, 3- extended LD segments, 4- elevated admixture contribution from one population (MALD), 5- differentiating between populations measured by FST. The low diversity, and the high linkage disequilibrium observed from genotypic data of strains from the flor cluster, did not enabled us to apply methods 1 to 4. The detection of differentiation between populations based on an Fst scan

has enabled the detection of specific sites in different studies (Akey et al., 2010; Akey, Zhang, Zhang, Jin, & Shriver, 2002; Amato et al., 2009; Moradi, Nejati-Javaremi, Moradi-Shahrbabak, Dodds, & McEwan, 2012) and could have provided as well an alternative. We indeed tested this approach, but it did not provide any clear signal in our hand, again very likely because of a too strong population structure, seen from the admixture output, from the long branch supporting the flor yeast cluster and from the high linkage disequilibrium between loci that may indicate a population with low proportion of sex.

Because of these difficulties we have chosen to use Principal Component Analysis (PCA), which enabled us to differentiate wine and flor yeasts and thus to detect regions that contribute to these differences. In a second step, we performed a genomescan of two parameters based on intraspecies polymorphism: nucleotide diversity π and Tajima's D, on a data set combining wine and flor strains in order to detect regions with the highest divergence between the two groups, which could attest from balancing selection.

We obtained two first datasets from PCA, lead on the whole set of SNPs and on the set of biallelic positions. These two sets shared 55 genes, and the 91 other genes specific to the whole set of variant pointed into genes contained by TY1 transposable elements, or repeated regions inside ORF, which explained the poor mapping of SNPs contained in these genes. However, for several genes such as *HXT3*, *PDC1*, *FRE3*, the poor mapping was caused by a high divergence of part of the coding sequence. For the first gene, a specific region inside the gene caused this drop in mapping, whereas for the two others this was caused by a recombination between genes of the same family e.g. *PDC1* and *PDC5*, *FRE3* and *FRE2*.

The three methods performed on the same set of SNPs provided a shared group of 45 divergent genes, containing two divalent metal transporters *ZRT1* and *SMF1*, proteins involved in signal transduction *RGA2*, *BUD2* or pseudohyphal growth *STE7*, *RGA2*, *DBR1*. If we take in account the 312 features obtained by each method (about 5% of *S. cerevisiae* ORFs) one can be surprised by the number of them which were already described to have a role in velum formation: *IRA1* and *IRA2* for the Ras/cAMP/PKA signalling pathway and *HKR1*, *RGA2*, *STE7* for the MAPK signalling pathways and, *SLN1* for the HOG pathway. It has been reported that Hog1 and Hkr1, the MAP kinase and osmosensor of the HOG

MAPK pathway, inhibit the filamentation pathway (Pitoniak et al., 2009; Cullen and Sprague, 2012). Thus we can postulate that *SLN1* also could regulate *FLO11*.

The large number of divergent genes between flor and wine strains that are involved in divalent metals transport suggests that metal homeostasis is important for flor and wine strains. These genes include *ZRT3* (the vacuolar transporter of zinc inducted under low zinc conditions), *SMF1* and *ALR1* (manganese and magnesium transporters respectively), but also *TRE2* that functions with Tre1p to regulate ubiquitylation and vacuolar degradation of Smf1p, or *UBC7* which is involved in resistance to cadmium poisoning reinforce this picture. Last, the *FRE2-3* genes, encoding ferric reductases, were found recombined whereas *FRE6* was also found specifically divergent from any other origin. In addition, the analysis of highly impacted genes enabled us also to detect a possible truncated protein coded by *AFT1* in the genome of flor yeast.

A comparison of flor strain P3-D5 and wine strain K1 transcriptome enabled us also to highlight some of the specificities of flor yeast.

The first pattern observed was the up regulation of *FLO11* and also other three flocculins: *FLO1*, *FLO5* and *FLO9*. But many other categories were also differentially expressed.
One first group of genes differentially regulated between P3-D5 and wine strain K1 are genes involved in metal homeostasis: *ZRT1*, *CUP1-1* and *CUP1-2* (two metallothioneins that bind copper and cadmium) were overexpressed in flor strain while some genes involved in iron uptake and *IZH4* (a membrane protein involved in zinc metabolism) were down regulated. More generally we could detect an overexpression of genes expressed under condition of zinc deprivation in the genome of flor strain (Cheraiti, Sauvage, & Salmon, 2008), whereas genes involved in iron import and under the control of Aft1 were down regulated. In addition, cytochrome subunits were found overexpressed in flor strain P3-D5 while genes associated to respiratory deficiency were found upregulated in wine strain K1. This can be associated to the necessary respiratory metabolism of flor strains to keep them at the wine surface (Jiménez and Benítez, 1988).

Other significant and interesting differences between flor and wine yeasts similarities were aso detected, such as transcriptional regulation by inositol (Santiago & Mamoun, 2003), that indicates a response of flor yeast to a deficiency in inositol**.** Another unexpected

feature was the overexpression of DNA helicases with telomerase recombination activity (*YRF1-1*/*2*/*3*/*4*/*5*/*6*/*7*).

Among the list of genes identified to differentiate flor and wine strains, we chose to further study the role of four target genes potentially involved in flor adaptation to biological ageing conditions: *PIK1*, *FRE2-3*, *PDC1* and *ZRT1*. For one gene, the hexose transporter *HXT3*, we could associate phenotypic differences to the allelic versions: this allele had previously been characterized in an industrial wine strain as a fructophilic version of the wild type transporter *HXT3*.

The locus exhibiting the highest divergence between flor and wine was the promoter region of *PIK1*, encoding a phosphatidylinositol 4-kinase. *PIK1* is an essential gene described to have different roles inside the cell and one of these was to regulate the activity of three yeast MAPK (Cappell and Dohlman, 2011) including Kss1, involved in the activation of *FLO11*. The analysis of the most mutated portion of *PIK1* promoter revealed the presence of several putative transcription factor binding sites: among them Nrg1, a transcriptional repressor of invasive growth and biofilm formation (Kuchin et al., 2002), present only in the wine promoter and Tec1, a transcriptional activator of most filamentation genes (Chou et al., 2006), present only in the flor promoter. A protein structure prediction showed some variations in the secondary structure, binding residues and ligands between flor and wine protein indicating that also mutations in the ORF could play a role in a possible different activity of flor Pik1, especially regarding velum formation. The allelic replacement of promoter and coding region of this gene will provide informations about a potential regulatory activity in biofilm formation.

Our second target *FRE2-3* genes, encoding two ferric reductases, showed a high number of translocations in flor strains and we did not manage to achieve the construction of the required mutant to validate this hypothesis. More work has to be done in order to evaluate the surrounding of *FRE3* and *FRE2* in flor strains and to clarify if these translocations correspond to advantageous mutations (in *FRE* genes or neighboring genes) or to loss of function of flor alleles.

Another recombined gene was *PDC1*, which encodes pyruvate decarboxylase. This gene carried the central domain of *PDC5* and we hypothesized that this translocation could result in an enhanced secondary function of the enzyme particularly useful during flor ageing: the detoxification of acetaldehyde, or could lead to the production of secondary alcohol, that may have an impact in quorum sensing (Wuster & Babu, 2010). The cloning of the two alleles of *PDC1* in a PDC minus strain enabled us to compare them, but did not provide any significant metabolic difference that could lead to an acquired secondary function of flor *PDC1* allele.

The last gene for which we tried to evaluate the phenotypic impact was *ZRT1*. We constructed *ZRT1* allelic replacement mutant strains and our attempts to detect a role in adaptation to biological ageing were also unsuccessful, in particular that zinc is the catalytic component of the superoxide dismutase *SOD1*. A wider screening realized on many growth media, performed in a phenotype microarray analysis did not provide us more information. Similarly, Engle and Fay (2013) were not able to detect different phenotypes caused by the allele of *ZRT1* during fermentation. Several authors reported that *ZRT1* not only has the function of carrying zinc inside the cell but could also influence the transport of other metals, such as cadmium (Gitan et al., 1998; Gomes et al., 2002; Gitan et al., 2003), and the allelic differences may be related to the transport of other metals and not only zinc. However, transcriptomic results as well as the numerous genes found mutated in flor strains strengthen our hypothesis that metal homeostasis has a key role in velum formation or wine fermentation. The finding of the role of phosphatidylinositol (4,5) diphosphate in invasive growth (Guillas et al., 2013) suggests a stronger role of phosphoinositols, and maybe of Pik1, in velum formation. As zinc availability participates to the regulation of phosphoinositols synthesis, the global zinc deficiency detected in the transcriptome of flor strain suggests also a role via a requirement in phosphoinosides. The construction of a double replacement mutant *ZRT1-PIK1* and its phenotypic impact under low zinc conditions might shed light on the role of these genes in biofilm formation.

Flor and wine yeast domestication

The pattern of these numerous mutations raises the question of yeast domestication. This question is ancient, raised by Martini (Martini, 1993) and brought back to light by Fay and Benavides (Fay and Benavides 2005). The diversity of wine yeast is undoubtedly lower

than that of a collection of isolates obtained world wide from different resources, but is still lower than that of a cluster of American natural isolates characterized from a wide collection of yeast with RAD-tag sequencing (Cromie et al., 2013) which indeed is a good argument for domestication.

What is domestication? Domestication can be considered as a co-evolutionary interaction leading to the establishment of new domesticated species, the growth and reproduction of which are mostly controlled for the benefit of another species (Meyer & Purugganan, 2013). Do wine yeast follow that model? The first population genomic study lead on *S. cerevisiae* (Liti et al. 2009) revealed also that the "European/wine cluster" contains wine and soil or clinical isolates suggesting that these strains are not limited to the winery environment. And indeed, the question of wine yeast life cycle along the year is still unclear. Cellars produce huge populations of *S. cerevisiae* strains during wine making periods and can be a source of inoculation for the vineyard. But as well vineyard isolates have been sampled, often at low frequencies from vine from berries or barks or insects i.e. (Capece et al., 2012; Cromie et al., 2013; Goddard et al., 2010; Mortimer & Polsinelli, 1999; Schuller & Casal, 2007; Schuller et al., 2012; Setati et al., 2012; Stefanini et al., 2012; Valero et al., 2005), and these studies have shown that cellar microflora is related to vineyard microflora, and that vineyard microflora should be seen as a complex ecosystem in which insects (such as wasp, bees, or drosophila) or birds (Francesca et al., 2012) participate to yeasts dispersal. Wine yeast are very well adapted to this ecosystem: their efficiency during fermentation (Camarasa et al., 2011) and the frequent detection of translocation between chromosome VIII and XVI leading to a higher of the expression *SSU1* gene (Pérez-Ortín et al. 2002) as well as a non-neutral evolution of its transcription factor *FZF1* (Aa et al., 2006), attest of that. In brief some "domestication genes" have been identified for wine yeast, but how far man controls yeast growth and life cycle is still questionable given the complexity of vineyard ecosystem. In contrast, as proposed earlier (Legras et al. 2007; Sicard and Legras 2011) flor yeast represent a very good example of adaptation to a man driven environment: the mutations of the *FLO11* gene enabling the production of a velum required for velum growth and selected among flor strains is a typical "domestication gene" as seen for plants and is a good argument in favor of domestication. Recently, Zörgö et al. (2012) showed that life history traits shapes the genetic make up of the different lineage through the accumulation of loss of function

alleles, pointing out the rarity of adaptive alleles. In our case, we can suspect the accumulation of loss of function alleles (i.e. *LTO1*) leading to the deregulation of *FLO11*. For other genes their contribution to the specific phenotypes of flor and wine lifestyle is still unclear.

Hitherto, our functional evaluation of genes especially divergent between flor and wine strains failed in the detection of domestication alleles. This does not refute our method but means that more work is necessary to find phenotypic evidence that target genes are involved in the adaptation to biological ageing conditions.

5. CONCLUSIONS AND PERSPECTIVES

This study provided new insights about flor strains phylogeny, in particular their relationship with wine strains, and also about EC1118, which appeared as a cross between a flor and a wine strain. The comparison of the genome sequences of flor and wine strains enabled us to detect several genes with allelic variation that may explain flor yeast adaptation to Sherry-like wines biological ageing. These can be classified in three main groups: a group of genes involved in metal transport, a group of genes involved in *FLO11* regulation, and genes with potential metabolism impact such as *HXT3*, *PDC1*, *ARO10*.

A transcriptome analysis comparing a wine and a flor strain revealed some of the specificities of flor strains: up regulation of floculin genes (*FLO1*, *5*, *9*, *11*), differential regulation of genes involved in metal homeostasis (up regulation of genes expressed under zinc-limiting conditions and down regulation of genes involved in iron uptake in flor strain), overexpression of genes encoding cytochrome subunits and of DNA helicase with telomerase recombination activity. In addition flor yeast apparently would display higher requirement in inositol.

We detected a gene, *PIK1*, potentially involved in the regulation of *FLO11* and hence in biofilm formation. The analysis of the promoter portion highly mutated in this gene revealed the presence of potential transcriptional factor binding sites: Nrg1, a transcriptional repressor of biofilm formation, in wine strains and Tec1, a transcriptional activator of most filamentation genes, in flor strains. The prediction of protein structure showed some differences in the folding and binding residues of flor and wine proteins. The allelic exchange of promoter and coding region of *PIK1* will reveal the potential role of this gene in biofilm production.

For *FRE2* and *FRE3* genes, we did not manage to perform allelic replacements in flor strains. It will be necessary to evaluate the surrounding of *FRE3* in flor strains and to understand if these translocations correspond to advantageous mutations or to loss of function of flor alleles. While for *ZRT1* gene we were not able to detect a differential phenotype, we could detect differences for *PDC1* gene in the two strains suggesting that the wine allele has the highest activity. Further analyses have to be done to find the optimal conditions highlighting phenotypic differences. The construction of a double mutant *ZRT1-PIK1* may also be useful to identify the potential role of these genes in the adaptation to flor environment.

Other experiments now possible with the tools developed in this thesis could provide further insights into biological ageing. The search for QTL for velum growth using a progeny between a wine and a flor strain would help us to decipher the key genes leading to differences in velum development, and the transcriptomic analysis of flor strains during velum ageing would also offer an overview of actives genes during biological ageing. But, above all, a deeper analysis of genes targeted by this study should provide more genes involved in flor yeast domestication.

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