Chapter 24
Functional Genomics in Wine Yeast: DNA Arrays and Next Generation Sequencing

Ana Mendes-Ferreira, Marcel lí del Olmo, José García-Martínez, and José E. Pérez-Ortín

24.1 Introduction

The transformation of a grape juice into wine results from the biochemical activity of many microorganisms, particularly yeast. *Saccharomyces cerevisiae* wine yeast strains are able to completely ferment sugar-rich natural musts under conditions that other strains are unable to. Additionally, they are particularly well adapted to the harsh conditions of fermentation, characterised by high sugar content, high alcohol content, low pH, the presence of sulphites, copper, limiting amounts of nitrogen, anaerobiosis and other environmental stresses. For those reasons, *S. cerevisiae* is still referred as the wine yeast par excellence.

Over the last years, winemaking industry have benefit tremendously from the established interest of the scientific community in *S. cerevisiae* fundamental research, being a model organism for studies in cell biology, biochemistry and in molecular biology for many years. The sequence of the reference laboratory strain S288c entire genome was accomplished before any other eukaryote in 1997 (Goffeau et al. 1996, 1997), and since then about 420 laboratory, industrial and wild strains have been extensively annotated (Borneman and Pretorius 2015; Borneman et al. 2016; Gallone et al. 2016). Given the considerable genetic

A. Mendes-Ferreira (✉)
BioISI-Biosystems and Integrative Sciences Institute—UTAD, Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal
e-mail: anamf@utad.pt

M.l. del Olmo • J.E. Pérez-Ortín
Facultad de Biológicas, Departament de Bioquímica i Biologia Molecular, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Spain
e-mail: m.del.olmo@uv.es; jose.e.perez@uv.es

J. García-Martínez
Departamento de Genética, Universitat de València, Dr. Moliner 50, 46100 Burjassot, Spain
e-mail: Jose.Garcia-Martinez@uv.es

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information available, the leading role of this eukaryotic model has been evident in the development of powerful tools for analysis. For instance, global gene expression studies by means of microarray analysis were first performed using *S. cerevisiae* (Schena et al. 1995; Wodicka et al. 1997; DeRisi et al. 1997; Hauser et al. 1998) and proved to be instrumental in the unravelling of the complexity of gene expression regulation under several conditions. Moreover, it has been continuously being improved because this yeast is the working horse for the development of different technical improvements (Hughes et al. 2001; García-Martínez et al. 2004; David et al. 2006). Logically, these DNA array studies were first done in laboratory strains of *S. cerevisiae* growing in laboratory conditions. These strains do not exhibit the same properties as industrial strains, and the growth conditions are significantly different; therefore, their responses may be quite different. However, rapidly this genome-wide approach received a strong interest in the subsequent years to address the question of the adaptation of industrial wine yeasts to the actual winemaking conditions. This review presents a synopsis of DNA array and next-generation sequencing (NGS) technologies and focus mainly in their use in studying wine yeast gene expression profiles, recapitulating the major findings about *S. cerevisiae* biology that have emerged from its application and how they contributed to the improvement of industrial winemaking process. Although the use of microarrays to generate gene expression data has become widespread, thanks to the advent of NGS, RNA-seq has recently become an attractive alternative method in the studies of transcriptomes, promising several advantages compared with microarrays.

24.2 Short Overview of the DNA Array Technology

By definition "array" means "to place in proper or desired order". A DNA array (also commonly known as gene or genome chip, DNA chip or gene array) is a collection of DNA spots, commonly representing single genes arrayed on a solid surface (glass, plastic, silicon chip or nylon) by the covalent attachment to chemically suitable matrices or simply by electrostatic binding. The immobilised DNA segments are known as probes, and many thousands can be placed in known locations on a single DNA microarray (see Fig. 24.1 for a schematic representation of DNA chip technology).

DNA arrays can be fabricated using a variety of technologies, including printing with fine-pointed pins onto either glass slides or nylon membranes, photolithography using pre-made masks, ink-jet printing or electrochemistry on microelectrode arrays. By regarding the printing surface and the technology used for fabrication and processing, different kinds of DNA arrays can be distinguished:

Spotted Microarrays The probes are cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto a glass surface. This type of array is typically hybridised with cDNA from two samples to be compared and is labelled

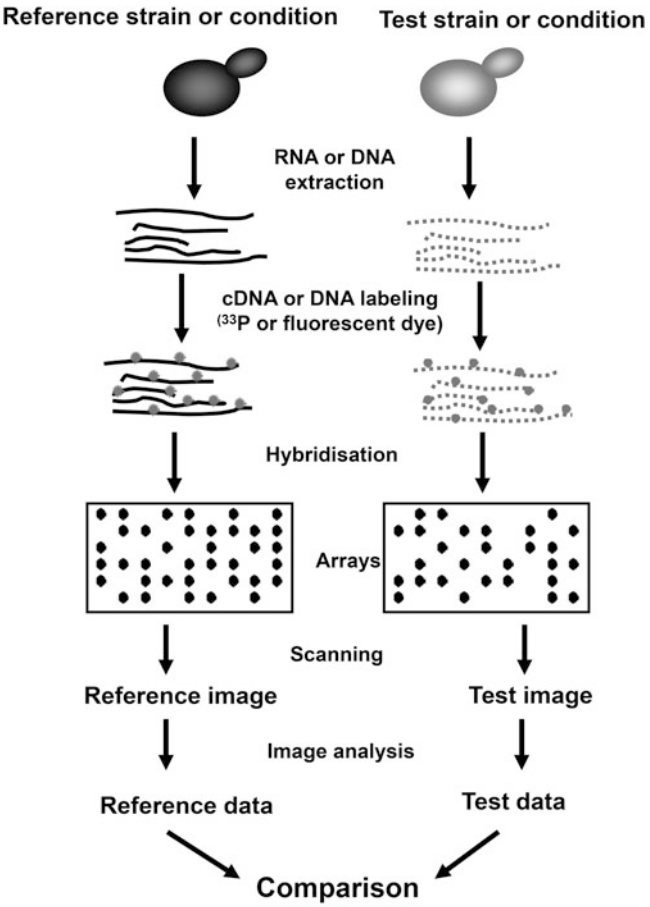

Fig. 24.1 Schematic representation of the different steps in the DNA array processing and analysis. Note that in the hybridisation step in macroarrays and in some kinds of oligonucleotide arrays, two independent hybridisations are performed whereas in most glass microarrays, both test- and reference-labelled samples, are hybridised simultaneously on the same slide

with two different fluorophores. The two labelled cDNA samples are mixed and hybridised to a single microarray that is then scanned to visualise the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore signal may then be used in ratio-based analysis to identify upregulated and downregulated genes. Absolute levels of gene expression cannot be determined in the two-colour array, but relative differences in the expression among different spots (=genes) can be estimated.

Spotted Macroarrays Equivalent to the previous one but in which the probes are immobilised onto a positively charged nylon membrane. mRNA is radioactively labelled (usually ^{33}P). Each condition (e.g. wild type and mutant) is hybridised

independently with a stripping step between them, which allows the use of the same arrays for different sample replicates.

Oligonucleotide Microarrays The probes are designed to match parts of the sequence of known or predicted mRNAs. There are commercially available designs that cover complete genomes from different companies. These microarrays can provide estimations of the absolute value of gene expression. Oligonucleotide arrays can be either produced by piezoelectric deposition with full-length oligonucleotides or by *in situ* synthesis. Long oligonucleotide arrays are composed of 50–60 mers and are produced by ink-jet printing on a silica substrate. Short oligonucleotide arrays are composed of 25–30 mer and are produced by photolithographic synthesis on a silica substrate or piezoelectric deposition on an acrylamide matrix.

Genotyping Microarrays They are spotted macro- or microarrays than can be used to identify genetic variation in individuals and across populations. In this array, the labelled genomic DNAs from the strain to be tested along with the reference strain S288c are competitively hybridised to a spotted array containing probes of each gene of the later. The comparison of the signal intensities of both strains is then associated with the enlargement or deletion of genes in the tested strain relative to the reference. Short oligonucleotide arrays can be used to identify the single nucleotide polymorphisms (SNPs) that are thought to be responsible for genetic variation.

Tiling Arrays They are a kind of microarray that includes overlapping oligonucleotides designed to blanket the entire genome each 5–20 nucleotides without any previous knowledge of the coding regions. They can be used either for genotyping or expression studies.

24.3 Impact of DNA Array Technology on Yeast Gene Expression Research

The availability of the *S. cerevisiae* genome sequence has led to the discovery of many gene sequences but not their function. Since then, many functional analysis projects have been dedicated to the investigation of the molecular biology of this yeast, making use of omic tools developed based on genome knowledge. The first of these high-throughput techniques, DNA arrays, provided one entry point for functional genomics, changing the paradigm of gene expression analysis that has been limited to small number of genes (Lockhart et al. 1996). Global expression analyses have helped to elucidate their role in both cellular physiology and the way in which their mechanism works. The first studies compared expression patterns of one third of the yeast genome in different metabolic states (Lashkari et al. 1997). The advances in the array-based techniques allowed the expression of approximately 6000 genes of the yeast *S. cerevisiae* grown under a few different conditions to be

monitored on a single chip (DeRisi et al. 1997; Wodicka et al. 1997), using probes and primers obtained or designed from the laboratory strain S288c sequence. While Wodicka et al. (1997) compared gene expression in yeast cells grown on rich and minimal media, other pioneering comprehensive studies characterised the genes that were differentially expressed during the shift from fermentation to respiration (DeRisi et al. 1997; ter Linde et al. 1999; Kuhn et al. 2001), during sporulation (Chu et al. 1998), during the cell cycle (Cho et al. 1998; Spellman et al. 1998) or in response to conditions or treatments of interest, such as chemical or environmental agents (Jelinsky and Samson 1999; Jelinsky et al. 2000). In a landmark experiment that studied yeast response to 13 varied environmental conditions (Gasch et al. 2000), the authors found that while some genes altered its expression in a particular condition, a large set of genes showed a similar response to almost all the conditions studied, being generally termed environmental stress response (ESR) genes. The authors actually found that while some genes alterations were specialised for specific stresses, a large set of genes (the ESR ones) showed a similar response to almost all the conditions studied. This ESR share features with the previously recognised general response to stress, comprising a set of ≈50 genes induced by a variety of stresses through the stress response element (STRE) promoter sequence and recognised by the transcription factors Msn2p and Msn4p (see Estruch 2000 for a review). The majority of 900 ESR genes are repressed in response to acute stresses and are involved in growth-related processes including ribosomal protein genes, along with the large set of genes involved in RNA metabolism and protein synthesis. On the other hand, approximately 300 genes are induced in the ESR and involved in a wide variety of processes, including carbohydrate metabolism, detoxification of reactive oxygen species, cellular redox reactions, cell wall modification, protein folding and degradation, DNA damage repair, fatty acid metabolism, metabolite transport, vacuolar and mitochondrial functions, autophagy and intracellular signalling (Gasch et al. 2000). Later, it has been revealed that the ESR is not only a transcriptional response, but it also encompasses a post-transcriptional (mRNA stability) response that contributes to the fine adjustment of the induction and repression peaks (Canadell et al. 2015). More recently, it has been shown that there are differences in environmental stress response among yeast species with the more pronounced differences mostly found in the induced genes, whereas the repressed ones are highly conserved (Brion et al. 2016).

Other major work in this field discerned the function of regulatory proteins, such as transcription factors or subunits of transcription complexes, and either studied the consequences of overexpression or examined mutants (DeRisi et al. 2000; Holstege et al. 1998; Kobor et al. 1999; Myers et al. 1999; López and Baker 2000; Lee et al. 2000; Sudarsanam et al. 2000; Carmel-Harel et al. 2001).

For yeast biologists, the main achievement of the early gene expression studies was the discovery of genetic regulatory mechanisms, providing data to link genes and pathways to phenotypes in such a way that components of any metabolic and regulatory pathway could be determined. The wealth of data provided by the microarrays allowed the formulation of hypotheses that could be tested with other more traditional experiments. On the other hand, genome-wide expression

experiments on yeast validated the wide application of the technology and led to the development of a variety of other genome-scale technologies, which allowed mapping the binding sites of transcription factors in vivo by chromatin precipitation followed by DNA microarray (ChIP-chip) (Horak and Snyder 2002), analysis of screens of pooled mutants (Giaever et al. 2002; Pierce et al. 2007), quantification and detection of distinct spliced isoforms (Clarck et al. 2002) or genome-wide assessment of transcription rates (García-Martínez et al. 2004).

In the pursuit of a more comprehensive understanding of yeast physiology and metabolism, along the last years, numerous large-scale functional genomics studies have been performed, and *S. cerevisiae* response to different perturbations has been investigated. Presently, there are 1371 and 398 gene expression experiments hosted by public gene expression databases such as Gene Expression Omnibus (Edgar et al. 2002; Barrett et al. 2013) and ArrayExpress (Brazma et al. 2003; Kolesnikov et al. 2015), respectively. Restricting the search for wine yeast, we found fewer transcriptomic studies in both databases, 60 and 9 experiments, respectively. The development of the Saccharomyces Genome Database (SGD) (Cherry 2015) was essential for collecting, organising, storing and accessing the data from yeast large-scale studies. Also the curating of the data derived primarily from focused studies to generate machine-readable Gene Ontology (GO) annotations for yeast genes (Ashburner et al. 2000) turned possible for the yeast scientific community to address the roles of previously uncharacterised genes and to map novel functional connections between seemingly unrelated processes (Boone 2014).

24.4 Impact of RNA Sequencing on Yeast Gene Expression Research

As denoted above, transcriptome analysis by DNA arrays has played a central role in yeast functional genomics unravelling the complexity of gene expression regulation. Nevertheless, it is acknowledged that this methodology suffers from several caveats. For instance, for the construction of DNA microarrays, it is mandatory to have prior knowledge about genome sequence of the organism being studied. Up to 37,000 SNPs can be found when comparing laboratory strains (Schacherer et al. 2007), and the problem can become even more complicated for non-laboratory yeast strains. Indeed, in the sequence comparison of a wine strain AWRI1631 to S288c, an SNP frequency of 1 per 150 base pairs or roughly 7 SNPs per kilobase was found (Borneman et al. 2008). Also, microarrays often cannot readily distinguish closely related sequences due to cross-hybridisation jeopardising specificity and the quantification of RNAs expressed at a low level. On the other hand, saturation of spot signals puts an upper limit on the amount of expression that can be reliably quantified. To address these last two limitations, real-time qPCR is commonly used to validate microarray-generated data (Chuaqui et al. 2002; Brazma et al. 2001). Finally, there are several different microarray platforms

commercially available and other DNA arrays produced in-*house* using completely different probe sets which turn difficult the comparison of the data generated. Indeed, most of cross-platform comparisons are done by analysing each platform data set independently using the most appropriate normalisation method and statistical tests for each, and only afterwards the lists of significantly differentiated genes are compared.

In this line, next-generation RNA sequencing (RNA-seq) has recently become an attractive method in the studies of transcriptomes. Briefly, total or fractionated RNA is converted to a library of cDNA fragments with attached adaptors which are then sequenced. These reads are aligned to a reference genome or transcriptome set and can be counted to determine differential gene expression (Nagalakshmi et al. 2010) (see Fig. 24.2 for a schematic representation of RNA-seq technology). An RNA-seq protocol, covering yeast RNA extraction, cDNA synthesis, cDNA fragmentation and Illumina cDNA library generation with some brief remarks on bioinformatics analysis, is presented by Waern et al. (2011). This technique provides several advantages compared with microarrays. For once, RNA-seq does not depend on prior knowledge of sequence as RNA-seq labelled cDNA in parallel and multiple times. Also, cross-hybridisation and range of detection are not a concern since there is no hybridisation step involved, and due to the digital nature of RNA-seq, there is an unlimited dynamic range of detection (reviewed in Wang et al. 2009). While surpassing the mentioned microarray disadvantages, in order for RNA-seq technology to reach its full potential, a number of experimental and computational challenges need to be addressed, including the handling of read mapping uncertainty, sequencing non-uniformity, estimation of potentially novel isoform (alternatively spliced transcript) expression levels and efficient storage and alignment of RNA-seq reads (Li et al. 2010).

Again, *S. cerevisiae* was one of the first species in which transcriptome reconstruction RNA-seq was evaluated (Nagalakshmi et al. 2008). In that study, the authors revealed the transcriptional landscape of the yeast being able to detect novel sequences through de novo assembly of sequences that did not match with the reference genome. RNA-seq yielded a comprehensive view of both the transcriptional structure and the expression levels of transcripts showing that nearly 75% of the non-repetitive sequence of the yeast genome is transcribed (Nagalakshmi et al. 2008; Wang et al. 2009). Besides *S. cerevisiae*, RNA-seq has already been applied to other yeast species including *Candida albicans* (Bruno et al. 2010), *Candida parapsilosis* (Guida et al. 2011), *Candida glabrata* (Linde et al. 2015), *Schizosaccharomyces pombe* (Bitton et al. 2015), *Cryptococcus neoformans* (Toh-E et al. 2015), *Pichia anomala* (Fletcher et al. 2015), *Pichia pastoris* (Valli et al. 2016), *Brettanomyces bruxellensis* (Capozzi et al. 2016) and *Kluyveromyces marxianus* (Schabort et al. 2016). On the other hand, only a few studies have been carried out with this technology in biofuels (McIlwain et al. 2016), Chinese rice wine (Li et al. 2014) and baker's (Aslankoohi et al. 2013) and wine (Treu et al. 2014b; Nadai et al. 2015, 2016, see below) industrial *S. cerevisiae* yeasts.

RNA-seq has proven to be extremely powerful and continues to advance raising the question about the future of microarrays technology in gene expression studies.

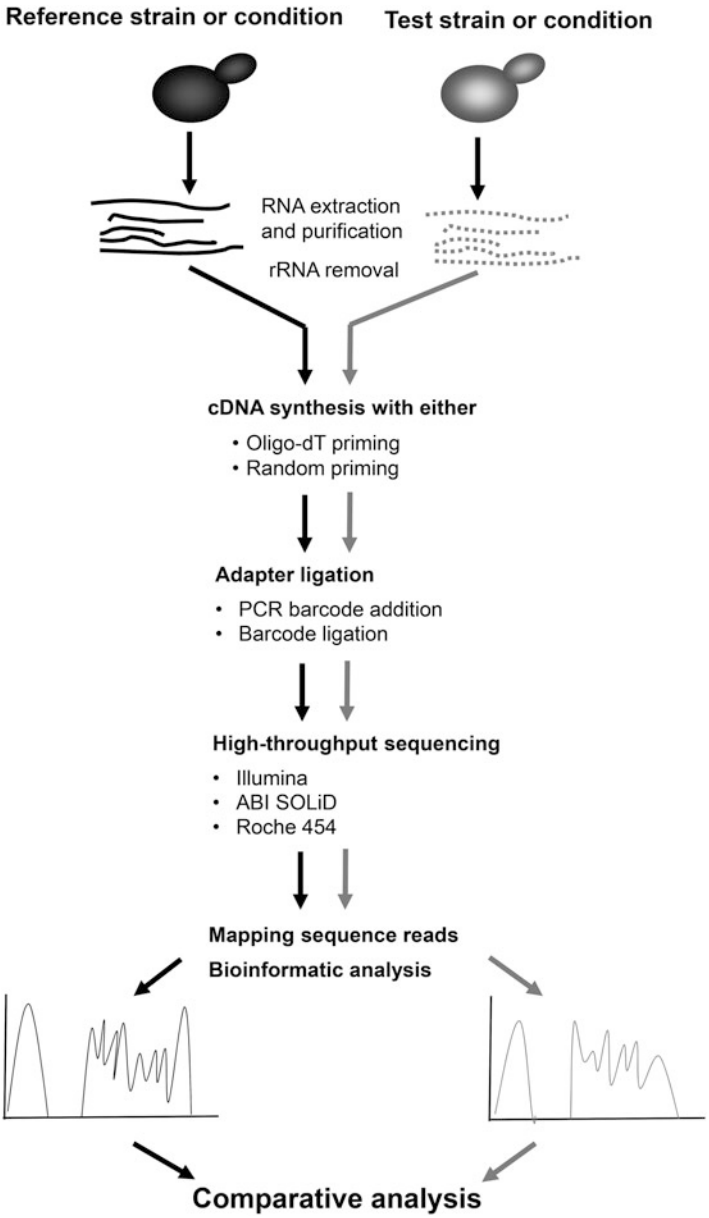

Fig. 24.2 Schematic representation of the different steps in the NGS processing and analysis

Recently, Nookaew et al.(2012) presented the first comprehensive comparison of both methods for analysis of transcriptome data of *S. cerevisiae* using the laboratory strain CEN.PK113-7D grown under two different metabolic conditions: respiro-fermentative (batch) or fully respiratory (chemostat) metabolism. Their results

underlined the importance of accurately mapping the reference genome to estimate gene expression level and to identify differentially expressed genes. Nevertheless, the authors found high consistency between microarray and RNA-seq platforms. More recently, a single extraction of mRNA from *S. cerevisiae* was quantified by both microarrays and RNA-seq in parallel (Robinson et al. 2015). In this study, they multiplexed each lane of RNA-seq profiling so that it exactly mirrored the eight-array per chip design of the microarray platform that was used. The authors concluded that microarrays, while more consistent in their estimates across technical replicates, may show systematic biases at low intensities that confound differential expression detection suggesting that low-expressed genes of special interest should be monitored cross-platform. Taken together, both studies encourage the continual use of microarray as a versatile tool for differential gene expression analysis. In some way, RNA-seq technology will certainly contribute to the improvement of microarrays; actually, as new sequences are discovered, they could be incorporated in the *S. cerevisiae* arrays increasing their coverage, keeping microarrays relevant. In addition, this technology can boost the development of arrays for other biotechnological important yeast species which has been limited by the lack of sequence information available.

24.5 Transcriptional Response of *Saccharomyces cerevisiae* to Oenological Relevant Stresses

The transformation of grape juice into wine is accomplished by the activity of several microorganisms, mainly yeasts that are responsible for conducting alcoholic fermentation. During winemaking, yeast strains come across acidic pH (2.9–3.6), hyperosmotic stress due to the high sugar concentration in musts (up to 260 g/L), low nitrogen content and the presence of inhibitors such as sulphite, occasionally low temperature and, later, anaerobiosis, nitrogen starvation and high ethanol concentration (up to 15% v/v) (reviewed in Attfield 1997; Pizarro et al. 2007), being selected based on their ability to adapt to this harsh environment. Although there is a great variety of wine-related yeast species harboured in the skin of grapes, *S. cerevisiae* is still referred as the "wine yeast" mostly due to its stress resilience and unequalled fermentative ability, being able to adjust and completely ferment sugar-rich natural musts under conditions that other strains are unable to (Camarasa et al. 2011). The impressive adaptation of these wine strains to the oenological environment is related to variation in gene expression, as a consequence of genetic differences, either on coding or non-coding regions (Salinas et al. 2016) with regard to other *S. cerevisiae* strains of different origins (Cavalieri et al. 2000; Fay et al. 2004; Wang et al. 2007; Carreto et al. 2008), and in some cases correlates with the niche from which the strains have been isolated (Warringer et al. 2011). Recently, whole genome sequencing performed on 196+19 wine strains of *S. cerevisiae*, including commercial and natural isolates, indicated that these strains contain

relatively little genetic variation compared to the global pool of *S. cerevisiae* diversity (Borneman et al. 2016; Gallone et al. 2016, see below).

Unlike the genome sequence, the transcriptome is very dynamic with genes being high or lowly expressed according with the external stimulus. DNA microarrays have been extensively used to study yeast molecular responses to stress situations. The already cited study by Gasch et al. (2000) on a laboratory strain was used to elucidate how *S.* cerevisiae yeast cells respond when exposed to 13 different environmental stresses including osmotic shock amino acid starvation, nitrogen depletion, progression into stationary phase and oxidative stress which are relevant in the winemaking context.

Also the molecular responses of *S. cerevisiae* exposed to various wine-relevant stresses, including osmotic shock (Kaeberlein et al. 2002; Jiménez-Martí et al. 2011), ethanol (Alexandre et al. 2001; Fujita et al. 2004; Hirasawa et al. 2007; Lewis et al. 2010), sulphite (Park and Hwang 2008), nutrient limitation (Boer et al. 2003; Pizarro et al. 2008), acclimatisation to low temperature (Leng Tai et al. 2007) and CO_2 pressure (Aguilera et al. 2005), have been also addressed. Logically, these DNA array studies were mostly done in laboratory strains of *S. cerevisiae* growing in laboratory conditions. The inclusion of wine yeasts in some of these studies lead to the uncovering of some transcriptomic and genomic differences between wine and non-wine yeast strains. For instance, in the T73 wine yeast strain in relation to oxidative metabolism, *YHB1*, a gene encoding a flavohaemoglobin, whose expression is elevated in aerobic conditions in laboratory strains (Liu et al. 2000), is only slightly expressed in wine yeast. A small deletion found in its promoter is thought to be the reason (Hauser et al. 2001). This event may reflect the physiological features of the wine strain, which has been evolving for billions of generations under the almost anaerobic conditions of wine fermentation. Also, genes involved in sulphur (*SUL1-2*) and ammonia (*MEP2*) transport (Cavalieri et al. 2000) or that involved in sulphite resistance (*SSU1*) were found to be highly expressed in wine yeast strains (Hauser et al. 2001). The overexpression of these genes might be a developed detoxification strategy giving the continuous contact of these strains with copper sulphate and sulphur dioxide, used in controlling mould growth on grapes or in preservation during the winemaking process, respectively. In this line, Pérez-Ortín et al. (2002a) investigated in great detail the possible mechanisms for the expression regulation of the *SSU1* gene of the T73 wine yeast strain. A rearrangement of the promoter of *SSU1* was detected and led to an upregulation in its expression. We concluded that human involvement and the traditional vinification protocols led to a selection of wine yeasts which resist these agents. Also, Aa et al. (2006) analysed both the population genetic variation and population structure of *S. cerevisiae* by sequencing the coding region of *SSU1* and three other loci (*CDC19, PDH1, FZF1*) in 27 strains from very different locations in Italy and Pennsylvania, collected from oak forests and vineyards. The phylogenetic reconstruction showed the existence of differences between oak strains and wine strains, indicating that differences within *S. cerevisiae* populations are more likely due to ecological factors than to geographic factors. Recently, NGS and quantitative trait loci (QTL) mapping have discovered a different reciprocal chromosome translocation involving *SSU1*

promoter that increases sulphite resistance in other wine yeast strains (Zimmer et al. 2014). The high sequence polymorphism found in the *SSU1* gene suggests the existence of a diversifying selection on its protein product, thus supporting our previous proposal of a strong selection for this gene during the historical use of sulphur-based fungicides in winemaking. Additionally, it is known that wine strains diverge on their susceptibility to sulphite (Barbosa et al. 2014). Recently, Nadai et al. (2016) using RNA-seq to study strain-dependent SO_2 resistance have confirmed the main role of Ssu1p transporter in SO_2 tolerance and its importance in discriminating resistant from sensitive strains. Also, the *CUP1* gene, which is related to copper resistance (Karin et al. 1984; Winge et al. 1985), was found to be less expressed in YPD in the T73 wine strain than in the S288c background (Hauser et al. 2001). This could be due to a small deletion in the *CUP1* locus region (Pérez-Ortín et al. 2002b) or a higher number of copies of *CUP1* among wine strains compared with other isolates (Almeida et al. 2015). Recently, a promoter variant of *CUP1* with increased expression variability was identified in the wine yeast strain EC1118 conferring improved resistance to environmental stress conditions (Liu et al. 2015).

Also, growth temperature was found to lead to differential transcriptional responses among laboratory (CEN.PK113-7D) and wine (EC1118) strains of *S. cerevisiae*, centred on genes involved in sugar uptake and nitrogen metabolism (Pizarro et al. 2008). The levels of expression of both the low-affinity transporter *HXT1* gene and the high-affinity transporter *HXT6* and *HXT7* genes were higher in the wine yeast than in the laboratory strain. On the other hand, the authors showed that the levels of expression of high-affinity nitrogen transporters and amino acid biosynthetic genes were higher in the laboratory strain, whereas in the wine yeast, there was increased transcription of anabolic and catabolic genes involved in nitrogen metabolism, suggesting that the laboratory yeast is more starved for nitrogen than the wine yeast.

Differences in the genome-wide expression profile between laboratory (W303 diploid) and wine strains have also been found when exposed to osmotic stress caused by high sugar concentrations (Jiménez-Martí et al. 2011). The authors associated the improved adaptability of the ICV16 wine yeast, as seen by the higher percentage of viable cells and increased ability to grow in 20% of glucose, with the higher expression of genes related with amino acid and nucleotide metabolism (particularly biosynthesis), glycolysis, alcohol and ergosterol metabolism and DNA replication. In this sense Pizarro et al. (2008) observed higher expression of genes associated with the cellular response to nitrogen starvation in the laboratory strain used in their study (CEN PK113-70) when compared with the wine counterpart EC1118. In a comparative genome hybridisation on array (aCGH) study (see later), Carreto et al. (2008) reported that among the genes depleted in five commercial wine *S. cerevisiae* strains, relative to the reference strain S288c, were four copies of tandemly repeated cell-wall asparaginase genes (*ASP3-1*, *ASP3-2*, *ASP3-3* and *ASP3-4*), which are induced in response to nitrogen starvation. Taken together, these studies reinforce the suggestion that nitrogen metabolism is differentially regulated among these strains.

In sum, these studies have shown that although wine and laboratory strains are genetically highly related, the genetic basis of their distinct technological properties under fermentation conditions is still largely unknown. Recently RNA sequencing performed on the four vineyard strains, as well as on the industrial wine yeast strain EC1118 and on the laboratory strain S288c, revealed that *cis* and, more significantly, *trans* variations have a markedly different effect on transcriptional variability among strains with the latter being the major determinant of the fermentation characters that differentiated the strains examined (Treu et al. 2014a). Nevertheless, the data acquired in the studies using laboratory strains allowed a better understanding of the molecular mechanisms underlying yeast stress response and paved the way for the identification of gene targets or gene expression patterns that allow industrial yeast strains to adapt to each particular condition. The use of standard laboratory conditions enabled the comparison of specific metabolic and physiological features of natural isolates or commercial wine yeast strains in relation to the laboratory strains. However, those experiments in which the cells are transiently exposed to a single stress at a time do not efficiently reproduce the natural environment for wine yeast considering the dynamic succession of stresses occurring along the winemaking process.

24.6 Expression Responses of Wine Yeasts to Stress Situations During Vinification

Until the development of DNA microarray analysis, some traditional gene expression studies including only a small number of genes were conducted with wine *S. cerevisiae* yeasts. The first gene expression study in wine yeasts was conducted on a haploid strain, V5 (a non-usual wine strain) by Northern blot analysis of 19 genes which had been previously described as being expressed in laboratory growth conditions or on molasses during the stationary phase and/or under nitrogen starvation. Nine genes, including members of the *HSP* family, showed a transition-phase induction profile (Riou et al. 1997). A more comprehensive study was conducted on the same haploid wine strain and on a reference strain FY69 (S288c background) by the same group. In this case, 99 genes from chromosome III were studied by Northern blot analysis (Rachidi et al. 2000). A particular wine strain, T73, isolated from Alicante wines (Querol et al. 1992), has been selected in our laboratory for the expression studies of particular sets of genes. A molecular study using Northern blot was conducted on it (Puig and Pérez-Ortín 2000a, b). The expression patterns of glycolytic genes, and of nine other genes that were characterised by DeRisi et al. (1997) as showing a peak of induction at the diauxic shift, were studied. The T73 strain (and other commercial wine yeast strains) has also been useful to demonstrate the relevance of the expression of genes involved in the response to osmotic stress (mainly *GPD1*, encoding the glycerol-3-phosphate dehydrogenase gene) during the first hours of vinification (Pérez-Torrado et al. 2002;

Zuzuárregui et al. 2005). Gene expression analysis have also been carried out along benchtop trials of industrial wine yeast propagation in order to identify stress responses that might be relevant for the performance of active dry yeasts. After testing the expression profiles of a selected set of stress gene markers, the induction of the stress responsive gene *TRX2* during the batch stage of industrial growth suggests that an oxidative stress response can occur at the transition from fermentative to respiratory metabolism (Pérez-Torrado et al. 2005).

Partial transcriptomic analysis with commercial wine yeast strains, which differ in their fermentative behaviour, has also helped to understand these differences and to obtain clues to understand the best adaptation of several strains. Our research groups have carried out several analyses in this sense. A first study limited to two commercial strains and several well-characterised stress-responsive genes (HSP family and others) showed that *HSP12* could serve as a molecular marker for stress resistance in wine yeasts (Ivorra et al. 1999). Later on, analyses of this kind with 14 oenological strains demonstrated that it is possible to establish a correlation between stress resistance and fermentative behaviour (Zuzuárregui and del Olmo 2004a). Besides, although each strain shows a unique pattern of gene expression (Carrasco et al. 2001), higher (and in some cases maintained) mRNA levels of many stress genes tested were found in the strains with severe fermentative problems (Zuzuárregui and del Olmo 2004b), which suggest the requirement of and accurate stress response during vinification.

24.7 Genome-Wide Expression Studies in Wine Yeast

As previously mentioned, the natural environment of *S. cerevisiae* has shaped the evolution of this organism's metabolism to allow it to exploit the harsh winemaking environment. From its inoculation into grape juice until the end of the fermentation process, *S. cerevisiae* is exposed to stress situations that are reflected in the yeast's gene expression pattern.

Inoculation of grape musts with active dry yeast is a common practice in wine industry. Little is known about the transcriptional changes occurring during the biomass propagation step used in the industrial production of dry yeast, but a transcriptomic and proteomic analysis carried out by Gómez-Pastor et al. (2010) revealed that the most critical step is the metabolic transition from respiration to fermentation-based growth. Its use requires a previous rehydration process in which yeast cells restore their cellular functions. Some studies have analysed the genomic response in commercial wine yeast strains to rehydration and adaptation to osmotic stress at the beginning of vinification. In the first study, rehydration was carried out in a complete glucose medium to identify events related to re-establishment of fermentation (Rossignol et al. 2006). The authors reported substantial transcriptional changes. The expression profile observed in the dried yeasts was characteristic of cells grown under respiratory conditions and exposed to nitrogen and carbon starvation and considerable stress during rehydration. Furthermore, many genes

involved in biosynthetic pathways (transcription or protein synthesis) were coordinately induced, while those subject to glucose repression were downregulated. While expression of general stress-response genes was repressed during rehydration, despite the high sugar levels, that of acid-stress genes was induced, probably in response to the accumulation of organic acids. In the second study, rehydration was carried out in water to separate this process from adaptation to osmotic pressure (Novo et al. 2007). The results of the study showed that rehydration for an additional hour (following an initial period of 30 min) did not induce any relevant changes in global gene expression. The incubation of rehydrated cells in a medium containing fermentable carbon sources activates genes involved in the fermentation pathway, the nonoxidative branch of the pentose phosphate pathway, ribosomal biogenesis and protein synthesis. Also addition of the rehydration nutrient mix downregulated the expression of genes involved in the biosynthesis of different amino acids and vitamin/cofactor transport, consistent with its composition in these nutrients (Winter et al. 2011). Previously, Erasmus et al. (2003) analysed yeast response to high sugar concentrations by inoculating rehydrated wine yeast in Riesling grape juice containing equimolar amounts of glucose and fructose to a final concentration of 40% (wt/vol) and comparing global gene expression with that observed in yeasts inoculated in the same must containing 22% sugar. Although the sugar concentration used is not generally found in winemaking conditions, some of the results coincided with those reported by Rossignol et al. (2003), with sugar stress resulting in the apparent upregulation of glycolytic and pentose phosphate pathway genes and structural genes involved in the formation of acetic acid from acetaldehyde and succinic acid from glutamate and the downregulation of genes involved in the de novo biosynthesis of purines, pyrimidines, histidine and lysine. The authors also reported considerable changes in the expression levels of stress-response genes. These changes affected, among others, genes involved in the production of the compatible osmolyte glycerol (*GPD1*) and genes encoding the heat shock proteins *HSP104/12/26/30/42/78/82* and *SSA3/4*. In agreement, Jiménez-Martí et al. (2011) by means of gene expression analyses with several wine yeast strains found that the higher expression of genes involved in both biosynthetic processes and glycerol biosynthesis was directly associated with the improved ability of yeasts to growth in grape juice.

Large-scale transcriptome monitoring during alcoholic fermentation under conditions mimicking an oenological environment was first reported by Rossignol et al. (2003) that analysed samples taken at different time points during fermentation of a synthetic must. The authors found genes involved in C-compound metabolism, mitochondrial respiration/oxidative phosphorylation, stress responsive genes and a large number of genes with no biological process associated [130 genes from various subtelomeric families of unknown function (*PAU, AAD, COS*)] to be induced during wine fermentation. On the other hand, genes primarily involved in cell growth, protein biosynthesis and ribosomal processing functions were repressed in response to stress associated with alcoholic fermentation progression. A common description of gene expression during fermentation of synthetic or natural grape juices has consistently been described, although with differences in

gene expression patterns between strains (Rossouw et al. 2008). The greatest effect on gene expression is produced upon entry into the stationary phase, probably explained by a cell proliferation arrest in response to nitrogen depletion, a process regulated by the TOR pathway (Rossignol et al. 2003). The changes in gene expression seen in this phase, however, appear to differ from those observed under laboratory conditions (Gasch et al. 2000). In a latter comprehensive study of the dynamics of the yeast transcriptome during wine fermentation, Marks et al. (2008) discovered 223 genes that were dramatically induced along the process. They called this the "fermentation stress response" (FSR). The most interesting point was that the FSR was found to overlap only partially with the ESR (Gasch et al. 2000). Interestingly, 62% of the FSR genes were novel, suggesting that the stress conditions in wine fermentation were rather different from those observed in laboratory conditions. Also of interest was the fact that respiratory and gluconeogenesis genes were expressed even in high glucose concentrations and that ethanol accumulation was the main reason for entry into the stationary phase.

The amount of available nitrogen is considered to be one of the main limiting factors for yeast growth in musts (reviewed in Mendes-Ferreira et al. 2011). Studies performed with wine yeasts have generally found high expression levels for genes linked to amino acid and purine biosynthesis (Backhus et al. 2001; Cavalieri et al. 2000; Hauser et al. 2001), which are indicative of high growth rates. Activation of the methionine biosynthesis pathway and alterations in sulphate and nitrogen assimilation are known markers for metabolic phenotype as they are connected with cell-cycle progression (Patton et al. 2000). The effect of nitrogen availability on the growth of wine yeasts has been analysed in recent studies. One of these compared global gene expression profiles in synthetic media containing high and low concentrations of arginine (a source of nitrogen) (Backhus et al. 2001), whereas the other compared expression profiles in a Riesling must with normal concentrations of nitrogen and another to which diammonium phosphate (DAP) was added during the late fermentation phase, when yeast growth is no longer active (Marks et al. 2003). In the first study, it was found that nitrogen limitation induced genes that would normally be repressed by the high concentrations of glucose in the must. This suggests that, in the growth conditions that characterise the fermentation of must containing high concentrations of sugars and nitrogen, the use of glucose might be diverted, at least partly, to a respiratory metabolism (Backhus et al. 2001). This effect would be similar to what is known as the Pasteur effect, which is the inhibition of fermentation in the presence of oxygen. Although this effect has been reported to be irrelevant for yeast in laboratory growth conditions (Lagunas 1986), it might occur in the fermentation of musts with low levels of nitrogen, and, accordingly, cause sluggish or stuck fermentations. Curiously, they also found a slight increase in the expression level of genes encoding ribosomal proteins and those involved in ribosome biogenesis after nitrogen has been depleted. A more comprehensive and realistic study of transcriptional response in *S. cerevisiae* to different nitrogen concentrations during alcoholic fermentation was performed by Mendes-Ferreira et al. (2007a, b). The authors compared 11 samples from different time points of a series of control vinifications, nitrogen-limiting fermentations and

fermentations to which DAP was added. They found alterations in approximately 70% of the yeast transcriptome in at least one of the fermentation stages and also showed a clear association between these changes and nitrogen concentrations. In agreement with earlier findings published by Backhus et al. (2001), their results indicated that early response to nitrogen limitation involved the induction of genes associated with respiratory metabolism and a subsequent general decrease in the levels of genes associated with catabolism. More recently, our group (Barbosa et al. 2015a) performed a genome-wide study of the transcriptional response of three wine yeast strains with distinctive nitrogen requirements and fermentative profiles, under two contrasting nitrogen levels. This comparative transcriptomic analysis revealed common and strain-specific responses to nitrogen availability. In particular, domains of yeast metabolism related to nitrogen and sulphur (including amino acid metabolism and catabolism of nitrogen compounds) were heavily impacted at early fermentation stages by both differences in composition of fermentation medium and most importantly by the yeast strain. These differences were, at some extent, attenuate in latter fermentation stages, suggesting that the yeast strains may in fact alter the expression of a similar set of genes to cope with the stresses imposed during fermentation, but their adaptation to both nitrogen environments takes place in a different manner, in line with the specific fermentative and metabolic behaviour of each strain (Barbosa et al. 2014). Similar conclusions have been reached by Treu et al. (2014b) while using RNA-seq to analyse the expression profile of four vineyard strains of *S. cerevisiae* having different fermentation performances and compared with those obtained for the industrial wine strain EC1118 and for the laboratory strain S288c. Accordingly, the analysis of the genes involved in fermentation stress response revealed a lower expression in strains characterised by low fermentation efficiency, particularly in the first fermentation phase evidencing the high variability of transcriptional profiles among different wine yeast strains and their connection with complex phenotypic traits, such as the fermentation efficiency and the nitrogen sources utilisation. In search for the genetic basis of such variability on yeast nitrogen requirement, Brice et al. (2014) using a QTL approach identified four polymorphic genes (*GCN1*, *MDS3*, *ARG81* and *BIO3*) associated with differences in fermentative activity in a medium in which nitrogen was limiting.

The most common strategy used by winemakers to avoid premature fermentation arrest and to avoid the risk of sulphur off-flavours production is the addition of nitrogen compounds, such as DAP. A study by Marks et al. (2003) found that the addition of DAP affected the expression of 350 genes. The 185 genes that were found to be downregulated encoded small-molecule transporters and nitrogen catabolic enzymes, including enzymes involved in the synthesis of urea, which is a precursor of ethyl carbamate. The other 165 genes affected were all upregulated. These included genes involved in the biosynthesis of amino acids, purines and ribosomal proteins (suggesting a more active metabolism despite an absence of cell proliferation) and assimilation of inorganic sulphate (necessary for the elimination of hydrogen sulphide). The results of the study by Marks et al. (2003) provided a possible explanation for why the addition of DAP reduces the production of ethyl

carbamate and hydrogen sulphide, two undesirable components in wines. Similar results were later obtained by Mendes-Ferreira et al. (2007a) who found that the main transcriptional effect of adding DAP to a nitrogen depleted medium was an upregulation in genes involved in glycolysis, thiamine metabolism and energy pathways. A study performed by Jiménez-Martí and del Olmo (2008) showed that the effect of nitrogen refeeding depended on the source of nitrogen used, as they detected differences in gene expression reprogramming depending on whether ammonia or amino acids were added. The addition of ammonia resulted in higher levels of genes involved in amino acid biosynthesis, whereas that of amino acids directly prepared cells for protein biosynthesis.

Genome-wide expression analysis has emerged as a powerful tool for identification of genes that behave in a similar trend in a particular condition. The identification of genes that specifically respond to a specific stimulus (molecular biomarkers or signature genes) could be important for refining or complementing the existing diagnostic procedures. The genome-wide analysis performed on the yeast strain PYCC4072, growing in nitrogen-replete and nitrogen-depleted conditions, led to the identification of a set of 36 genes as promising candidates for prediction of problematic fermentations due to low nitrogen (Mendes-Ferreira et al. 2007b). A list of 46 potential nitrogen-dependent genes under winemaking conditions were also uncovered by Barbosa et al. (2015a), with a special emphasis to *CAR1*, *ATF1*, *DUR1,2 and PUT1*, which displayed the higher upregulation and to the ORF with unknown function, *YML057C-A,* which was the most downregulated gene under limitation of nitrogen. The fact that in that study we have used three contrasting yeast strains in gene expression analysis prompts this biomarkers identification more reliable, accurate and reproducible. Ethanol stress is another major pressure that *S. cerevisiae* has to deal with during vinification. Ethanol tolerance is still not fully understood, but it is known to partly depend on alterations in the plasma membrane (Alexandre et al. 1994). Global gene expression studies have provided a better understanding of the molecular basis underlying yeast response and resistance to ethanol stress (Alexandre et al. 2001; Fujita et al. 2004; Hirasawa et al. 2007; Lewis et al. 2010) under laboratory conditions. Using microarray analysis to identify target genes and analyse ethanol sensitivity in knockout strains, Hirasawa et al. (2007) found that the biosynthesis of tryptophan can confer ethanol tolerance. In our laboratory, we have studied the yeast response to sudden ethanol addition. A laboratory strain stops growing when ethanol is added to 7.5%. Growth is reassumed after several hours. At that time, a specific increase in the level of mRNAs of genes encoding cell wall components, hexose transporters and enzymes for carbohydrate metabolism is seen (Antúnez and Pérez-Ortín, unpublished data). Despite the data available from the global analysis of ethanol response in yeast laboratory strains, there are no published papers in which this topic is considered in wine yeasts. Usually wine strains are much more ethanol resistant than laboratory ones. Particularly, the flor yeasts involved in the biological ageing of sherry wines should cope with ethanol concentrations above 15% (Aranda et al. 2002). In this line, Lewis et al. (2010) have shown extensive natural variation in the response to acute ethanol stress among yeast strains while studying the

transcriptional response of a lab strain S288c, vineyard isolate M22 and oak-soil strain YPS163 exposed to ethanol. While targets of the "general stress" transcription factor Msn2p, the oxidative stress factor Yap1p and the proteasome regulator Rpn4p were all affected coordinately across the strains, thousands of gene expression differences in response to ethanol have been found.

The global transcriptomic studies conducted with wine yeast strains during alcoholic fermentation (Backhus et al. 2001; Rossignol et al. 2003; Marks et al. 2008), although not specifically devoted to ethanol stress, provided some insights into the topic, particularly the stress caused by progressive ethanol production. For instance, Backhus et al. (2001) and Rossignol et al. (2003) found changes in the levels of the expression of genes involved in biosynthesis of fatty acids, phospholipids and ergosterol during vinification. Genes encoding enzymes involved in the synthesis of fatty acids, phospholipids and ergosterol are highly expressed (Backhus et al. 2001) in *S. cerevisiae* yeasts but decrease towards the stationary phase. The results of the fermentation monitoring study conducted by Rossignol et al. (2003) indicated that anaerobic stress is a characteristic of wine fermentation and that the absence of ergosterol synthesis, one of the main growth-limiting factors for yeasts in musts with low oxygen and high ethanol levels, is due to the continuous decrease in the expression levels of genes involved in ergosterol biosynthesis. In agreement, while studying gene expression changes in *S. cerevisiae* at the late stage of very high gravity (VHG) fermentation, Zhang et al. (2012) found *ERG7*, *ERG20*, *ERG1 and ERG8* being highly repressed. On the other hand, only 5% of short-term ethanol stress genes (Alexandre et al. 2001) were found among the FSR genes which are considered to mediate long-term adaptation to the increasing ethanol levels, suggesting that ethanol activates unidentified ethanol signal transduction pathway which regulates FSR response (Marks et al. 2008).

Fermentation temperature is also an important factor in winemaking. For instance, white and rose wines fermentations are usually conducted at lower temperatures (12–17 °C) than red wines (22–28 °C) in order to reduce the volatility of aromatic compounds improving the sensory quality of wine. Global gene response of the wine strain QA23 has been analysed in fermentations carried out at 13 and 25 °C (Beltrán et al. 2006). The authors observed that the lower temperature induced cold stress response genes at the initial stage of fermentation and increased levels of genes involved in cell cycle, growth control and maintenance in the middle and late stages of fermentation. Furthermore, several genes involved in mitochondrial short-chain fatty acid synthesis were found to be overexpressed at 13 °C compared to 25 °C. These transcriptional changes were correlated with higher cell viability, improved ethanol tolerance and increased production of short-chain fatty acids and associated esters. Similar conclusions were obtained in a more recent study conducted in similar conditions (12.5 and 25 °C) but using different strains and grape juice varieties (Deed et al. 2015). Additionally, this comparative study found, along with changes in the cell wall and stress response, genes linked to three key nutrients to be strongly influenced by low temperature fermentation: nitrogen, sulphur and iron/copper. In agreement, García-Ríos et al. (2014) using an integrative approach, combining genomics, proteomics

and transcriptomics, reported that the upregulation of genes of the sulphur assimilation pathway and glutathione biosynthesis has a crucial role in the yeasts adaptation at low temperature. Only 137 genes out of 787 (17%) identified by Deed et al. (2015) were in common with those expressed in the cold wine fermentation performed by Beltran et al. (2006) confirming the strain specificity of the cold stress response between *S. cerevisiae* strains (García-Ríos et al. 2014), as seen for other stress conditions (Treu et al. 2014b; Barbosa et al. 2015a).

Under industrial conditions wine is obtained by a microbial consortia possessing various metabolic activities. Even in inoculated fermentations, there is a substantial yeast and bacterial biodiversity observed on grapes and musts that can persist during the fermentation process. The understanding of the microbial interactions that may occur during winemaking and how they affect the composition and quality of wines obtained are far from being known. A limited number of recent studies, involving lactic acid bacteria, have indicated that genome-wide transcriptome analysis can provide a better insight into the nature and molecular basis of microbial interactions (bacteria-bacteria) in mixed cultures of industrial organisms (Sieuwerts et al. 2010; Maligoy et al. 2008; Hervé-Jimenez et al. 2008). More recently, *S. cerevisiae* genome-wide transcriptional profiling in mixed culture has also been conducted to assess yeast-bacteria interaction, using *Lactobacillus delbrueckii* subsp. *bulgaricus*, which co-occur in kefir fermentations (Mendes et al. 2013) and cocultivated with the wine malolactic bacterium *Oenococcus oeni* (Rossouw et al. 2012). In this last work, the transcriptome of a commercial yeast strain in single and in co-inoculated fermentations with *O. oeni* was evaluated. This analysis showed that a significant number of genes were differentially expressed in *S. cerevisiae* under these two conditions. While genes involved in stress response, sulphur metabolic pathway, lipid biosynthesis and nutrient uptake were overexpressed in the co-inoculated fermentations, genes encoding for sterol biosynthesis and metabolism of phosphorus, proline and glycine were downregulated.

A first attempt to study yeast-yeast interaction in mixed culture wine fermentation using transcriptome-based approach has been carried out by our group using DNA arrays (Barbosa et al. 2015b). In that study, transcriptome profiling on mixed-culture fermentations was performed at three different time points, in mid-exponential growth phase (24h), in early stationary phase (48h), and in late stationary growth phase (96h), and compared to single *S. cerevisiae*-culture fermentations. We have detected a large set of genes that were differentially expressed that were associated to the presence of *Hanseniaspora guilliermondii* during fermentation confirming the importance of such a global approach for the study of yeast-yeast interactions during fermentation. The observed changes in the expression level of genes associated with vitamins biosynthesis and amino acid uptake and biosynthesis confirmed the nutritional interactions revealed or at least suggested by growth-based methodologies including competition for vitamins (Bataillon et al. 1996; Medina et al. 2012) and for nitrogen available (Fleet and Heard 1993; Medina et al. 2012) on grape must. The transcriptomic analysis carried out in our study were only performed in *S. cerevisiae* since microarray analysis is limited to organisms with sequenced genomes. The global response to mixed-culture growth in

H. guilliermondii remains to be established. Furthermore, it remains to be understood if these adjustments are specific to this strain or more generally linked to the presence of any competing yeast. In recent years, however, the development of high-throughput sequencing techniques such as RNA-seq has been successfully used to characterise the transcriptome of other wine non-*Saccharomyces* strains. Accordingly, RNA-seq approach was recently used to identify genes differently expressed after exposure to SO_2 in *Brettanomyces bruxellensis*, considered to be the main spoilage yeast in red wines (Capozzi et al. 2016). Global transcriptional analysis revealed that entrance and recovery of viable but non-culturable SO_2-induced state are associated with yeast sulphite toxicity and the consequent oxidative stress response.

The application of DNA array technology to wine strains has extended the landscape of expression studies. The studies on wine yeast using DNA array analysis have used various approaches in relation to growth conditions as well as the experimental design of the assay. Thus, whereas some experiments simulate the vinification conditions, by growing the wine yeast strain on a chemically defined synthetic must in an attempt to increase reproducibility and study particular stresses (Backhus et al. 2001; Rossignol et al. 2003; Zuzuárregui et al. 2006; Mendes-Ferreira et al. 2007a; Jiménez-Martí and del Olmo 2008; Rossouw et al. 2008; Jiménez-Martí et al. 2011; Carreto et al. 2011; Brice et al. 2014; García-Ríos et al. 2014; Orellana et al. 2014; Barbosa et al. 2015a), others used natural grape juices in their studies which are far more complex and variable (Marks et al. 2003, 2008; Erasmus et al. 2003; Beltrán et al. 2006; Deed et al. 2015; Barbosa et al. 2015b). In addition, we find a great heterogeneity among these studies in terms of the volume of fermentation used in the experiments. Thus, the studies conducted by F. Bauer group on the applicability of those experiments performed in synthetic medium to study conditions experienced in industrial fermentations and on the prospective extrapolation of the results obtained in small-scale laboratory fermentations to large-scale industrial environments were very pertinent (Rossouw and Bauer 2009; Rossouw et al. 2012). To answer the first topic, the transcriptomes of two phenotypically diverging commercial strains in two simulated wine must or real grape must (Colombard) at three stages of wine fermentation were analysed (Rossouw and Bauer 2009). The authors showed that gene regulation throughout fermentation, either on synthetic or real grape musts, did not differ significantly concluding that synthetic musts are indeed a valid model of real grape must fermentations. Later, Rossouw et al. (2012) used the same comparative transcriptomic approach assessing the response of an industrial wine yeast strain in parallel fermentations of a natural grape juice in small-scale laboratory (80 mL) and large-scale industrial conditions (110 L). Again, the authors found that yeast gene expression profiles in both conditions followed the same trend, concluding that small-scale fermentations in synthetic must are valid experimental models for investigation of microbial biology in real commercial fermentation processes. Most importantly, both studies validate the usefulness of all transcriptomic studies performed in *S. cerevisiae* towards the understanding of industrially relevant

aspects of winemaking that could be used by winemakers to improve the fermentation process and the quality of wines obtained.

24.8 Structural Genomics Studies in Wine Yeast Strains

Since the developing of NGS technologies, the whole genome sequencing of wine yeast strains has been applied to an increasing number of natural and commercial isolates (reviewed in Borneman and Pretorius 2015). These kinds of analyses have allowed to discover the similarities and differences of wine yeast genomes and to develop hypothesis on the origin and evolution of those strains (revised in Pérez-Ortín and García Martínez 2011). Several studies have recently investigated the diversity of *S. cerevisiae* species by sequencing the genomes of hundreds of different strains, providing a first glimpse of the complex evolution of this species (Almeida et al. 2015; Liti et al. 2009; Schacherer et al. 2009; Gallone et al. 2016). Indeed, from the comparative genomic analysis of up to 196 wine strains of *S. cerevisiae*, Borneman et al. (2016) concluded that all appear to represent a highly inbred population containing relatively little genetic variation compared to the global pool of *S. cerevisiae* diversity. This conclusion has been reinforced by another study that showed that wine yeast group is phenotypically distinct from wild strains and stems from a limited set of ancestral strains that have been adapting to winery environments. In spite of this, wine yeasts group in just one clade much more homogenous than beer yeasts which have stronger hallmarks of domestication (Gallone et al. 2016).

In spite of the recent application of NGS to wine yeast, DNA arrays are still used because of their simplicity and relative low price, for a variety of genomic research applications: systematic characterisation of genes discovered by sequencing projects, identification of new transcripts, detection of aneuploidies or partial chromosome deletions, chromosomal rearrangements and identification of interesting QTLs, among others.

Allelic variations can be detected in any strain by analysing the patterns obtained by hybridising genotyping arrays with total genomic DNA (Winzeler et al. 1998). Array hybridisation is strictly dependent on the precise sequence of the target; therefore, changes in the genes may produce differences in signal intensity or even no signal at all. Point variations (SNPs) are, however, difficult to detect with long probe arrays. The use of oligonucleotide arrays is the only way to analyse allelic differences in detail. For instance, Primig et al. (2000) have shown that SK1 strain has more genetic variation as polymorphisms and deletions (34%) when comparing the S288c standard background with the W303 background (5%).

With full-length ORF PCR-product arrays, it is possible to make array aCGH and monitor chromosome aneuploidy or chromosomal segment duplications (Hughes et al. 2000). aCGH is a simple but powerful technique that allows gross-comparisons of genomes, using a reference strain. It allows to test differences in gene copy number, ploidy and gross-chromosomal rearrangements that are, in part,

responsible for different developmental, morphological and physiological characteristics of the industrial yeast strains, as already indicated. The first aCGH study was performed by Hauser et al. (2001) who found important differences between laboratory and wine strains when both expression and genomic hybridisation values for transposon (Ty) ORFs were analysed. The low expression of these ORF in the wine yeast strain seems to be due to the fact that the laboratory strain (S288c genetic background) has more copies of transposable elements (Ty1–Ty4) than the wine yeast strain. This factor, also shared by other industrial yeast strains such as brewer's yeast strains (Codón et al. 1998), agrees with the suggestion that a negative selection for transposon accumulation might exist in the wild for the Ty elements. Ty elements recently expanded in laboratory strains because they lack of the strongly competitive wine or beer fermentation environment (Jordan and McDonald 1999; Codón et al. 1998). Another difference found in that study was the different number of subtelomeric genes in the T73 wine strain. In fact, it has been found that subtelomeric regions are the most variable region in the *S. cerevisiae* (and specially in wine yeast strains) genome including not only different copy number of subtelomeric gene families but also wine strain-specific loci (reviewed in Borneman and Pretorius 2015). For instance, *FSY1* gene, encoding a H+/fructose symporter, was first identified as a member of the large multigenic strain-specific locus present in the EC1118 group of *S. cerevisiae* wine strains (Novo et al. 2009). The presence of this gene is thought to support active transport of fructose into the cell, a phenotypic trait that is lacking (perhaps lost during laboratory evolution) from most *S. cerevisiae* strains and is predictable to provide a selective advantage during wine fermentation.

Using aCGH technique, Infante et al. (2003) found that two prominent variants of *S. cerevisiae flor* yeast strains differ from one another in the DNA copy number of 116 genomic regions that comprise 38% of the open reading frames (ORFs). They also found that the majority of them correspond to a widespread amplification of genomic fragments. By analysing the different situations found, the authors suggest that the amplifications have been produced by gross chromosomal rearrangements (GCRs) mediated by identified hotspots (transposon LTRs, tRNAs, subtelomeric repeated sequences, etc.), helped by bursts of double-strand breaks (DSBs) mainly produced by both acetaldehyde and ethanol. One of the unique properties of *flor* yeast is the production and release of high amounts of acetaldehyde as a consequence of ethanol assimilation. Since some of the genes among those involved in these copy number variations have functions related to the specific phenotypes that are characteristic of *flor* yeast strains, one possible suggestion is that this mechanism is responsible for the adaptive evolution of these yeasts. Actually, two changes in *FLO11* (a large deletion in the promoter and another one in the coding region) differentiate *flor* yeast strains from other non-floating strains (Fidalgo et al. 2006). Recently, the aCGH profiles of six flor strains from Spain, Hungary, France and Italy were compared (Legras et al. 2014). This analysis revealed differences in the subtelomeric regions but disagree with the previous study (Infante et al. 2003) arguing that copy number variations in

subtelomeric regions are not enough to explain the flor yeast adaptation to its environment.

The aCGH technique also allowed Dunn et al. (2005) to analyse four commonly used commercial wine yeast strains. They assayed three independent isolates from each strain and compared them with laboratory strain S288c. All four wine strains displayed common differences with regard to laboratory strain S288c. Some may be specific to commercial wine yeasts. Slight differences inter- or intra-strain were observed, indicating that they are closely related and quite genetically stable. Among the variations, there are genes that code for transporter proteins (similarly to the case of *FSY1* gene described before). Moreover, genes exist that are involved in drug resistance (or detoxification). The authors not only propose a "commercial wine strain signature", comprising the genes whose copy number is altered in all the wine yeast isolates examined in relation to the S288c strain, but also suggest that the differences in the fermentation and organoleptic properties of the different strains may arise from a small number of genetic changes.

Finally, aCGH has been also used by the A. Querol group to analyse ploidy and genome identity in *S. cerevisiae* × *S. kudriavzevii* hybrids from beer and wine (Peris et al. 2012). They found that all hybrids share a common set of depleted *S. cerevisiae* genes, which also are depleted or absent in the wine strains previously studied, and the presence of a common set of *S. kudriavzevii* genes, related with their capability to grow at low temperatures. They also found chromosomal rearrangement events in the hybrid genomes, which differentiate two groups of wine strain originated by different rare-mating events.

24.9 Conclusions

DNA array technology has been widely used on wine yeast research. DNA arrays are currently much more feasible and straightforward and are providing more clues towards an understanding of the biotechnology process. They have been particularly important in the disclosure of why some yeast strains are able to perform winemaking whereas others are not, why some of them are more resistant to particular stresses, and how the evolution has modelled the genome of this organism. To date, transcriptomic studies undertaken in the vinification context have only been carried out with *S. cerevisiae* strains. NGS techniques such as RNA-seq have recently started, allowing the characterisation of the transcriptome of other wine non-*Saccharomyces* strains. This technology will provide important genomic and transcriptomic data on these yeasts that is expected to revolutionise the manner in which global regulatory responses and development of the yeast-yeast interactions throughout alcoholic fermentation will be analysed. This knowledge will be of great importance in the improvement of current winemaking technologies and the accompanying yeast strains.

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