***Saccharomyces cerevisiae* gene expression during fermentation of Pinot noir wines at**

industrially relevant scale

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Abstract

During a wine fermentation, *Saccharomyces cerevisiae* transforms grape must

through metabolic activities that generate ethanol and other compounds. Thousands of

genes change expression over the course of a wine fermentation to allow *S. cerevisiae* to

adapt to and dominate the fermentation environment. Investigations into these gene

expression patterns have previously revealed genes that underlie cellular adaptation to the

grape must and wine environment involving metabolic specialization and ethanol

tolerance. However, the vast majority of studies detailing gene expression patterns have

occurred in controlled environments that do not recapitulate the biological and chemical

complexity of fermentations performed at production scale. Here, we present an analysis of

the *S. cerevisiae* RC212 gene expression program across 40 pilot-scale fermentations (150

liters) using Pinot noir grapes from 10 California vineyards across two vintages. We

observe a core gene expression program across all fermentations irrespective of vintage

similar to that of laboratory fermentations, in addition to novel gene expression patterns

likely related to the presence of non-*Saccharomyces* microorganisms and oxygen

availability during fermentation. These gene expression patterns, both common and

diverse, provide insight into *Saccharomyces cerevisiae* biology critical to fermentation

outcomes at industry-relevant scales.

Importance

This study characterized *Saccharomyces cerevisiae* RC212 gene expression during

Pinot noir fermentation at pilot scale (150 liters) using production-relevant conditions. The

reported gene expression patterns of RC212 is generally similar to that observed in

laboratory fermentation conditions, but also contains gene expression signatures related to

yeast-environment interactions found in a production setting (e.g., presence of non-

Saccharomyces microorganisms). Key genes and pathways highlighted by this work remain

under-characterized, raising the need for further research to understand the roles of these

genes and their impact on industrial wine fermentation outcomes.

Introduction

Saccharomyces cerevisiae is most often the dominant fermentative organism during vinification. As a domesticated species, it has evolved specialized metabolic strategies to assimilate sugars in grape must and transform them into ethanol, thereby outcompeting other microorganisms during fermentation (1). During this process, S. cerevisiae encounters a dynamic stress landscape. In early fermentation, sources of stress include high sugar concentration (osmotic stress), low pH (acid stress), decreasing oxygen (hypoxia), the presence of other organisms that compete for nutrients or produce inhibitory compounds, and sulfur dioxide additions that are used to inhibit spoilage organisms. As fermentation progresses, nutrients become limiting (starvation), temperature may rise or be kept low (heat/cold stress), and ethanol concentrations rise (ethanol stress). Yet, through a coordinated gene expression response, S. cerevisiae adapts to these stresses and most often continues fermentation until the must is dry.

High throughput gene expression profiling (e.g., microarray and RNA sequencing) has offered a window into the metabolic strategies used by S. cerevisiae during fermentation to adapt and dominate fermentation environments. Previous research has reported expression changes in >2000 genes during fermentation (2–4). In early fermentation, this is marked by expression of gene products that support biosynthetic processes and acquisition of abundant nutrient resources (2, 3). As fermentation progresses, nitrogen limitation, phosphate limitation, and/or ethanol accumulation can trigger a transition to a non-proliferative state (i.e., stationary phase), which involves remodeling the gene expression program to support cellular adaptation to the changing environmental with continued metabolism (2, 3). Towards the end of fermentation, relief of

nitrogen catabolite repression (2) and increased expression of nitrogen recycling genes (2,

5) is observed, which can be accompanied by further remodeling of the translational

machinery and increased oxidative metabolism (5, 6). As ethanol concentrations rise

through the end of fermentation, a gradual transcriptome response to ethanol stress is also

observed (3). This response overlaps with, but appears distinct from, the environmental

stress response seen in laboratory yeast (2, 3, 7), which may be related to the multitude of

simultaneous stresses experienced by the yeast at the end of a wine fermentation (Bisson

2019). Indeed, the majority of genes with stress response elements in their promoter are

expressed at the end of fermentation (8).

Through the associated metabolic processes that consume and produce a multitude

of compounds, *S. cerevisiae* gene expression in response to environmental factors is related

to overall fermentation kinetics and wine sensory outcomes. For example, fermentations

can become sluggish or stuck when *S. cerevisiae* inadequately adapts to stresses

encountered in the wine fermentation environment (9). In addition, altered gene

expression likely underlies differential wine sensory characteristics in fermentations

conducted with different industrial yeast strains (10, 11). To impact wine quality, genetic

strategies have been applied in an attempt to alter the expression of flavor-associated

genes (12), which have achieved variable levels of success. Consequently, further study of

the *S. cerevisiae* gene expression program across fermentation is required to understand

the yeast-environment relationship and how these interactions may be controlled to alter

fermentation outcomes.

Given the importance of the yeast-environment interaction in determining gene

expression, a major consideration with respect to collecting such data is the fermentation

conditions used. To date, the majority of gene expression surveys have profiled

fermentations that deviate in one or more ways from the industrial conditions in which

most fermentations take place. For example, hundreds to thousands of liters of grape must

are fermented to wine at industrial scales, while milliliter to liter volumes are commonly

used in laboratory studies of gene expression (2–5, 13–16). Industry scale fermentations

also have different kinetics than lab scale fermentations (4, 15, 17), and are less aromatic

due to differences in hydrodynamics (15, 18). Similarly, dissolved oxygen differs at lab

scale from industrial scale (4), which can impact fermentation outcomes (19). Possibly

reflecting these different environments, at the end of fermentation the expression of key

genes involved in amino acid transport and other core metabolic processes have been

shown to differ between lab and industrial fermentations (4). Consequently, we propose

that the physical and chemical differences in lab versus industrial scale wine fermentations

are important factors to consider when analyzing gene expression patterns across

fermentation.

Another major consideration when conducting gene expression studies, is that most

studies investigate the fermentative capability of *S. cerevisiae* in monoculture using sterile

synthetic media or filter sterilized grape must (2–5, 12). These controlled studies are

important and allow connections between the media, gene expression, and wine outcomes

to be made (12), but do not recapitulate the complexity of a natural grape must that varies

in parameters like nitrogen composition, pH, and phenolic and elemental profiles (20–23).

In addition, these experiments lack the diverse grape must microbiome that is a

contributing component of wine fermentations (24–38). These are all parameters that will

shape the fermentation environment and the metabolic response of *S. cerevisiae*.

Inter-species interactions are a critical component of the fermentation environment

that informs the biology and behavior of *S. cerevisiae* during fermentation. It has been

shown that non-*Saccharomyces* yeast impact the metabolism of *S. cerevisiae* through direct

and indirect interactions (39–41), leading to faster resource acquisition by *S. cerevisiae* in

early fermentation and altered metabolism of vitamins and minerals (40–43). While

research is still needed to describe the impact of a diverse microbial consortia on *S.*

cerevisiae during fermentation (44, 45), it remains that industrial fermentations are not

sterile and involve diverse microorganisms (28, 34, 35, 37, 38). Even in fermentations

treated with sulfur dioxide (SO_2) to control microbial spoilage organisms, native fungi and

bacteria are metabolically active during fermentation (38, 46, 47). This makes profiling *S.*

cerevisiae gene expression amongst diverse microbial consortia important, as it will lead to

a better understanding of the principles that govern *S. cerevisiae* gene expression and

metabolism during fermentation.

Here, to begin to address the impact of an industrial wine fermentation environment

on *S. cerevisiae* gene expression, we incorporate the inherent variability found in industrial

fermentations and determine the *S. cerevisiae* RC212 gene expression program across

chemically and biologically diverse Pinot noir grape musts. Specifically, time series RNA-

sequencing was used to capture the gene expression profiles of RC212 during 40

inoculated primary fermentations at pilot scale (150 liters) using California Pinot noir

grapes from 10 vineyards across two vintages. Using this data, a core metabolic program

was defined during fermentation, which is well reflected by lab-scale fermentations, in

addition to gene expression patterns that deviate from expectation. In particular, we

observe altered gene expression that may be explained by the presence non-*Saccharomyces*

organisms and regulation of metabolic processes related to stress, oxygen, and redox

balance throughout fermentation. These observations suggest that the core genetic

programs uncovered by lab-based studies are detected in industry-relevant fermentations,

but production-based environmental factors induce other gene expression programs that

are layered on top of the core gene expression program. We expect that understanding

such variations in gene expression within a wine production-like environment will be key

to defining approaches that can be used to manage commercial fermentation outcomes.

Results & Discussion

Conditions and rates of fermentation

Pinot noir grapes were harvested from the same 10 vineyards in California during the 2017 and 2019 vintages for wine production at the UC Davis Teaching & Research Winery (**Figure 1A**). To standardize fermentations, grapes from the same Pinot noir clone and rootstock were harvested at the same ripeness (~24 Brix, total soluble solids as a proxy for sugar concentration). We sampled duplicate fermentations that used the grape material from each vineyard for a total of 40 fermentations (20 from each vintage) at industry-relevant scales using the same wine making protocol. Each fermentation was inoculated with the commercial wine strain *S. cerevisiae* RC212 and sampled to collect cells for gene expression analysis at 16 (exponential phase / early fermentation), 64 (stationary phase / mid fermentation), and 112 (decline phase / end of fermentation) hours post-inoculation (**Figure 1B**). While sampling times were standardized across fermentations, the rate of fermentation varied, resulting in samples being collected across a range of Brix values (**Figure 1C**). Differences in fermentation rates likely reflect diversity in the starting material and differential fermentation outcomes, which has also been demonstrated in sensory studies performed on wines produced from these vineyard sites in previous vintages (48).

A consistent whole-transcriptome remodeling occurs during fermentation independent of vintage

Using 3′ Tag RNA sequencing (3′ Tag-seq), we profiled *S. cerevisiae* RC212 gene expression at 16, 64, and 112 hours after inoculation from both the 2017 and 2019

vintages. Since these samples provided gene expression measurements across a multitude

of Brix values (**Figure 1C**), data from all 10 sites was combined and used to perform

differential expression along the continuous variable Brix. The resulting data defines a core

vineyard-independent gene expression program of RC212 during California Pinot noir

fermentations. Under this model, log_2 fold change values represent the change in gene

expression for each unit increase of Brix. Given that Brix decreases during fermentation, a

positive log_2 fold change corresponds to a gene that decreased in expression as

fermentation progressed, while a negative log_2 fold value corresponds to a gene that

increased in expression as fermentation progressed (see examples in **Figure 2A**). After

performing differential expression, we further intersected the differentially expressed

genes across vintages to determine consistent changes that were vintage-independent.

From this analysis, 991 genes decreased expression as Brix decreased, while 951 genes

increased expression as fermentation progressed (**Figure 2AB, Table S1**). Each vintage

also showed unique differential gene expression patterns, which may occur due to vintage-

specific differences in fermentation. However, we generated these data at different times

and applied newly developed methods (UMI barcoding, see methods) for sequencing the

2019 samples, and as such we suspect that the higher number of differentially expressed

genes in the 2017 vintage may reflect differences in sequencing data quality. Nonetheless,

the large fraction of shared differentially expressed genes suggests that a core gene

expression program is followed independent of vintage.

Of the genes differentially expressed in fermentation and shared across vintage,

many are known to function in wine fermentation and are central to yeast growth,

metabolism, and cell survival (**Figure 2B and C**, **Table 1**). A strong signature of growth

early in fermentation is observed that included cellular investment in ribosome biogenesis,

metabolism of lipids, purines, and amino acids, as well as cell division machinery (**Figures**

2C and S1-S2). These processes, coupled with enrichment of associated pathways involved

in RNA transcription and transport, reflect energy use for cell growth and proliferation

associated with log phase growth occurring in early fermentation. Further in fermentation,

changes in ribosomal machinery gene expression occurred, as reported in previous studies

(49) (**Figure 2C**), reflecting a transition to a non-proliferative metabolic state. Late in

fermentation this was accompanied by changes in gene expression linked to nutrient

limitation, altered metabolism, and entry into meiosis (**Figures 2C and S3-S4**), which

included gene expression patterns consistent with hallmark isoform switches in hexose

transporters and glycolytic enzymes that occur as concentrations of glucose or fructose

change (50) (**Figures 2B and 2C**). For example, *HXT1* encodes a low affinity glucose

transporter that was more strongly expressed at the beginning of fermentation when

glucose is abundant. *HXT4* has a high affinity for glucose and is expressed when glucose

concentrations are low (51), which is also observed in our data as *HXT4* expression

increased in late fermentation. Importantly, the pathways we have identified as enriched in

early and late fermentation align with expectations based on previous research and the

known biology of *S. cerevisiae* during fermentation (2–5, 13–16). This highlights the core

processes that previous research efforts have defined and provides confidence that the

analysis methods employed in these pilot-scale fermentations capture these biologically

important transitions.

Beyond these previously defined core gene expression patterns, gene expression

signatures indicative of less understood processes within these fermentations are also

observed, which may be linked to the industry-like environment these studies were

performed in. These patterns of gene expression included signatures of nutrient limitation

in early fermentation and polyol metabolism in late fermentation that were both consistent

with interactions with non-*Saccharomyces* organisms. We also find signatures of

concurrent hypoxic and anoxic metabolism that suggests differential availability of oxygen

for some yeast populations throughout fermentation. In association, we observe mounting

gene expression that is likely involved in mitigating oxidative and other stresses. Finally,

few vintage-specific differences can be found, but those we do identify highlight gene

expression patterns that could be linked to altered fermentation outcomes. We discuss

these observations below.

Nutrient limitation in early fermentation

While gene expression data supports logarithmic growth at 16 hours post-

inoculation (**Table 1, Figures S1-S2**), at this early timepoint there is also evidence for the

expression of genes that are typically up regulated in response to nutrient limitation. *PHO5*

and *PHO89* are phosphate transporters that are induced during phosphate starvation (52),

both of which are expressed in early fermentation, along with *PHO90*. Phosphate limitation

can cause stuck fermentations, as phosphate is critical for cellular function as a component

of ATP, nucleotides, sugars, lipids, and macromolecules such as proteins (49, 53). Given

that all these Pinot noir fermentations went to completion, and that the majority of glucose

was converted to ethanol after the 16hrs time point, induction of genes encoding

phosphate transporters early in fermentation is not likely associated with phosphate

starvation. Instead, it may be a response to the presence of non-Saccharomyces yeast, as

co-cultivation of *S. cerevisiae* with *Torulaspora delbrueckii* led to the induction of a gene

encoding a high-affinity phosphate transporter (PHO84) after only three hours of

fermentation (40). Enological co-culture of S. cerevisiae with organisms such as

Hanseniaspora guilliermondii and *Brettanomyces* has also been linked to induction of genes

involved in vitamin biosynthesis in fermentation (54, 55), which could be indicative of

increased nutrient competition and depletion of some nutrients early in fermentation. We

similarly observe induction of genes that encode enzymes involved in biosynthesis of B

vitamins in early fermentation, including BIO2 (biotin biosynthesis), RIB3 and RIB4

(riboflavin biosynthesis), PAN6 (pantothenate synthesis), SPE3 and SPE4 (pantothenic acid

synthesis), and MIS1 and FOL1 (folate biosynthesis). In addition, THI21 was induced, which

is involved in thiamine biosynthesis. As with phosphate, this may be related to the presence

of metabolically active non-*Saccharomyces* microorganisms that have been detected in all

of these fermentations (56). We expect that continued work using industry-like

fermentations across grape varieties and yeast strains, as well as controlled fermentations

using reconstituted microbial consortiums, will be critical for understanding the relevance

of these gene expression signatures to wine fermentation outcomes. If understood, such

interactions could potentially be addressed through timely nutrient additions to a

fermentation to achieve desired outcomes.

Evidence of varied gene expression patterns linked to oxygen exposure during

fermentation

A wine fermentation is generally regarded as an anaerobic process given that the

carbon dioxide (CO_2) produced as a byproduct of ethanol fermentation protects must from

dissolved oxygen (57). Yet, within anaerobia, there is an important distinction between

hypoxic (low oxygen) and anoxic (no oxygen) conditions. In a fermentation, it is expected

that molecular oxygen (O_2) is introduced into the grape must by handling processes,

including pump overs, that may introduce small amounts of dissolved oxygen into

industrial-scale tanks (58). Stratification within a fermentation may also expose local cell

populations to different oxygen environments leading to yeast cell populations undergoing

different anaerobic processes. Within our data, we found gene expression patterns

consistent with different populations of cells experiencing varied levels of oxygen exposure

during fermentation. For example, the yeast cell wall undergoes remodeling in response to

oxygen availability, which is accomplished in part by regulated expression of cell wall

mannoproteins encoded by *CWP1/CWP2, DAN1,* and *TIR1-TIR4* (59). Specifically,

expression of *DAN1* and *TIR1-TIR4* occurs reciprocally to expression of *CWP1* and *CWP2*,

with the *CWP* genes being expressed in aerobic conditions and *DAN1/TIR1-TIR4* in

anaerobic conditions (59). *DAN1* expression is known to be repressed in aerobic conditions

by four independent regulatory mechanisms (60). Interestingly, expression of both *CWP1*

and *DAN1/TIR1-TIR4* was observed in early fermentation samples. Similarly, in early

fermentations both *HYP2* and *ANB1* were expressed. These paralogous genes encode

translation elongation factor eIF5A and are part of a family of paired genes for which

oxygen induces the aerobic isoform and represses the hypoxic isoform (61). *HYP2* is

expressed under aerobic growth, while *ANB1* is expressed under hypoxic growth and is

tightly regulated by the presence of oxygen (62). Together, these gene expression patterns

indicate varied gene expression programs within yeasts that may be explained by differing

levels of oxygen exposure.

Among late expressed genes, oxygen-regulated paired isoforms were also

expressed, including *COX5A* and *COX5B* that encode a subunit of cytochrome *c* oxidase.

Modulated expression of these two isoforms allows *S. cerevisiae* to produce holoenzymes

with different catalytic properties in response to oxygen (63). *COX5A* expression declines

between 5-1 μmol/L O_2, and is undetectable below 0.25 μmol/L O_2 while *COX5B* is

undetectable until 0.25 μmol/L O_2 (61). Simultaneous induction of both transcripts at the

end of fermentation is again consistent with cells experiencing varied levels of dissolved

oxygen in fermentation (58). In contrast, of the oxygen-regulated isoform pair *CYC1* and

CYC7 (61), only expression of the hypoxic isoform *CYC7* was detected at the end of

fermentation. The break point between expression of isoforms occurs at a higher

concentration of 0.5 μmol/L O_2 for *CYC1*/*CYC7* than *COX5A*/*COX5B* (61), which may

indicate that dissolved oxygen levels did not exceed 0.5 μmol/L and therefore was not

permissive to expression of *CYC1*.

In late fermentation, induction of pathways like glycerol degradation and proline

metabolism that require oxygen were also observed. Glycerol is a compatible solute

involved in combating osmotic stress and redox balance and is primarily produced in early

fermentation (64). We found induction of *GCY1* which encodes a glycerol catabolic enzyme

used in micro aerobic conditions (65), as well as *RSF2*, a transcriptional regulator of genes

that encode proteins required for glycerol-based growth. Proline metabolism genes *PUT1*,

PUT2, and *PUT4* were also expressed at the end of fermentation. Although proline is an

abundant amino acid in grape must, it is a non-preferred nitrogen source of yeast and

requires oxygen to be metabolized (66). It was further observed that *PUT1* and *PUT2* were

induced in a sealed laboratory wine fermentation, but that proline was not metabolized

given the absence of oxygen (2). Expression of *PUT1*, *PUT2*, and *PUT4* is regulated by

nitrogen catabolite repression (67) and the presence of proline in the absence of other

nitrogen sources (68), but is not regulated by the presence of oxygen. Intracellular proline

accumulation also protects *S. cerevisiae* from reactive oxygen species associated with

ethanol-rich environments (69). While it possible that glycerol and proline were

metabolized in late fermentation with oxygen ingress, other processes like nutrient

limitation and oxidative stress may also explain the induction of these genes.

Taken together, our gene expression data raises various questions about a

distributed gradient of oxygen (hypoxia and anoxia) in the fermentation environment that

may induce varied gene expression across the cell population. This could lead to yeast sub-

populations undergoing varied metabolic outputs or having different levels of ethanol

tolerance due to the role of oxygen in these processes (70, 71). In the future, single cell

sequencing technologies combined with continuously monitored dissolved oxygen assays

may help resolve these questions. From a production perspective, in industrial

fermentations, even those that employ pump over systems and therefore maintain mixing

and better homogeneity, there is a gradient of dissolved oxygen in the fermentation tank

wherein oxygen concentration is higher toward the top of the vessel (58). This suggests

that heterogeneous gene expression profiles in response to oxygen would likely exist in

these environments too. This is also an important fact to consider, as oxygen additions

during fermentation are known to influence both fermentation and sensory outcomes. For

example, in late fermentation, a single oxygen pulse increases the rate of fermentation

mediated by ergosterol biosynthesis (70). Similarly, oxygen additions at different stages of

fermentation differentially impact wine aroma compound formation like volatile thiols and

esters; however, this appears to occur in a strain-dependent manner (71). This knowledge,

combined with the impact of oxygen addition on fermentation outcomes, raises the idea

that timely addition of oxygen may be a way to control fermentations rates and formation

of wine aromas, which would be a tool easily accessible to winemakers.

Mitochondria and fermentation

In late fermentation, our gene expression analyses find a striking enrichment of

pathways involved in mitochondrial biogenesis and function, as well as oxidative

phosphorylation (**Figure 2C**, **Figure S3-S4**). Substantial metabolic investment in

mitochondrial systems suggests a critical role for mitochondria late in fermentation.

However, what that role is remains unclear, as limited research has been conducted on the

mitochondria during enological fermentation, likely because of both low oxygen conditions

and the Crabtree effect in fermentative metabolism (72, 73). While some studies that

profile the transcriptome of primary fermentation either find no evidence for, or make no

comment on enrichment for oxidative metabolism at the end of fermentation, many studies

have found induction of mitochondrial genes, particularly those encoding oxidative

phosphorylation. This includes fermentations conducted under nitrogen limitation (6),

lipid limitation (74), and standard laboratory conditions (3). Interestingly, under lipid

limitation, oxidative phosphorylation was induced in the exponential phase of growth as

opposed to the end of fermentation (74). Given the role of membrane lipid composition in

combating ethanol-induced membrane permeability (75), and the accumulation of reactive

oxygen species during ethanol exposure (76), induction of the respiratory chain may

mitigate reactive oxygen species that are abundant at the end of fermentation. Nonetheless,

the recurrence of these gene expression patterns in our studies and previous laboratory

experiments suggest that cells are investing in mitochondrial systems during fermentation.

One potential reason for late induction of mitochondrial systems is that glucose-

limitation relieves the Crabtree effect. This may lead to induction of oxidative

phosphorylation genes that change metabolism in a nutrient-limited environment to one

that generates the largest amount of ATP per unit of glucose (77). In this way, an

investment in mitochondrial infrastructure during late fermentation may be a starvation

adaptation in which *S. cerevisiae* uses oxidative phosphorylation to harness the largest

fraction of energy possible from remaining carbohydrate sources. However, this strategy is

predicated on availability of molecular oxygen, which is required for the induction and

function of the respiratory apparatus (78, 79). A second reason for mitochondrial gene

expression may be related to the fact that meiosis and sporulation related genes were

enriched at the end of fermentation (**Figure 2C**, **Figure S3-S4**). Induction of meiosis likely

occurs to produce spores resistant to the challenges of nutrient limitation and stress (80).

Interestingly, mitochondrial biomass is a predictor for meiosis (81), and components of the

respiratory chain are required for initiation of sporulation (82), providing another

potential process that may underlie mitochondrial investment in late fermentation. Related

to this fact, a propensity for yeast to undergo meiosis at this stage of vinification underlies

fast adaptive genomic evolution of *S. cerevisiae* (83), suggesting this may be an important

acquired trait that allows yeast to successfully survive the wine environment.

Mitochondria also fulfill other critical roles in fermentation unrelated to respiration.

For example, mitochondria play a role in sterol uptake and transport under strictly

anaerobic conditions (84), and mitochondria quench reactive oxygen species especially

during ethanol stress (85). While we did not observe induction of specific genes related to

sterol biology and found induction of different genes related to reactive oxygen species

than those previously identified (see next section), these processes may also be linked to

increased mitochondrial gene expression. Regardless of the role played by mitochondria in

late fermentation, the striking and consistent induction of these genes in fermentations

signals that more research is needed to understand the role of mitochondria in

fermentation.

Thioredoxins and glutathione system activity throughout fermentation

The reducing environment of the cytosol in *S. cerevisiae* is key to various cellular

functions, including deoxyribonucleoside triphosphate synthesis and the elimination of

toxic compounds, including oxidants generated through cellular metabolism (86, 87). Key

to maintaining redox balance are the thioredoxin (TRX) and glutathione (GSH) thiol-

reductase systems. For example, proper redox homeostasis is required to maintain the

redox status of cysteine residues, which are essential for the function of numerous

enzymes, protein receptors, and transcription factors. Similarly, redox homeostasis within

cells aids to balance pools of reduced and oxidized pyridine nucleotide cofactors (NAD+/H,

NADP+/H) that are essential to numerous metabolic reactions. Reactive oxygen species

(ROS) can alter this redox balance causing oxidative stress and direct or indirect ROS-

mediated damage of nucleic acids, proteins, and lipids. While typically associated with

respiratory metabolism, ROS can be generated throughout fermentation, in particular by

superoxide anions and peroxides (76, 88, 89). ROS may also be created by acetaldehyde, an

intermediate in ethanol production (90).

In early fermentation, we see induction of genes involved in the thioredoxin system,

such as *TRX1* and *TRR1*. Expressed targets of *TRX1* included *RNR1-RNR4* (91), genes

encoding ribonucleotide-diphosphate reductase required for DNA synthesis and cell cycle

progression, as well as *MET16*, which encodes an enzyme required for sulfate assimilation

(92). We further observed genes encoding Trx1 target peroxidases (*TSA1*) and

peroxiredoxins (*AHP1*) constitutively expressed throughout fermentation along with

superoxide dismutases (*SOD1*, *SOD2*). An additional source of ROS are peroxisomes, which

may generate hydrogen peroxide in early fermentation via beta-oxidation of fatty acids.

CTA1, which encodes a peroxisomal catalase, and *ANT1*, which encodes a peroxisomal

transporter involved in beta-oxidation of fatty acids, were expressed in early fermentation.

A major factor used to maintain redox balance is NADPH, which provides reducing

potential for the thioredoxin system. It has been shown that metabolic intermediates in

glycolysis can be re-routed to the pentose phosphate pathway to generate NADPH in

response to oxidative stress (93–95). We found that the pentose phosphate pathway was

enriched among genes expressed in early fermentation (**Figure 2C**, **Figure S1**-**S2**), which

includes *GND1*, an enzyme that catalyzes NADPH regeneration and is required for the

oxidative stress response. Other expressed genes that encode enzymes acting downstream

of *GND1* in the pentose phosphate pathway included *RPE1*, *TLK1*, *TLK2*, and *TAL1*.

Central to the glutathione (GSH) thiol-reductase system is glutathione, an abundant

tripeptide conserved throughout eukaryotic and prokaryotic cells with a critical role in

redox control, but its physiological role is both diverse and debated (95). We observed that

genes encoding enzymes involved in the degradation (*DUG1* and *DUG2*), import (*OPT1*), and

biosynthesis (*GSH1* and *GSH2* in the 2017 vintage) of glutathione were expressed in early

fermentation. Additional generation of NADPH in early fermentation may be supported by

the transformation of isocitrate to alpha ketoglutarate via *IDP1* in the mitochondria, and

export via *YMH2*, as both genes were also expressed. Genes encoding aldehyde

dehydrogenases *ALD5* and *ALD6* are similarly expressed in early fermentation, both of

which may regenerate NADPH through the transformation of acetaldehyde to acetate. *ALD6*

is the dominant isoenzyme responsible for acetate production in wine (96).

We further observed induction of genes involved in glutathione-mediated ROS

mitigation in late fermentation. For example, a gene encoding cytosolic glutaredoxin

(*GRX1*) was expressed in late fermentation. Unlike glutaredoxins in other species

(e.g., mammals), yeast glutaredoxins do not function as deglutathionylase enzymes (97).

Instead, induction of *GRX1* increases resistance to hydroperoxides by catalytically reducing

hydroperoxides through glutathione conjugation and using the reducing power of NADPH

(98). In addition, the cytosolic peroxidase *GPX1* was expressed. *GPX1* uses both glutathione

and thioredoxin , in combination with NADPH, for reducing power (99). *GPX1* is known to

be expressed by glucose and nitrogen starvation (100), which coincides with peak peroxide

formation in yeast during wine fermentation (88). While our gene expression data support

a role for cytoplasmic glutathione during late fermentation, genes encoding mitochondrial

peroxidin (*PRX1*) and thioredoxin (*TRX3*) were also expressed. Prx1 buffers the

mitochondria from oxidative stress and is reductively protected by glutathione, thioredoxin

reductase (Trr2), and Trx3 (101). Taken together, these results suggest that cytoplasmic

and mitochondrial systems may be integral to combating increased oxidative stress at the

end of fermentation.

Glutathione is also important for maintenance of cellular function via other systems.

For example, methylglyoxal is a byproduct of glycolysis, a reduced derivative of pyruvic

acid, that may account for up to 0.3% of glycolytic carbon flux in *S. cerevisiae* (102). We

found that *GLO2*, an enzyme that catalyzes methylglyoxal degradation in a glutathione

dependent manner, was expressed in late fermentation, as were glutathione-independent

systems involved in the degradation of methylglyoxal (*GRE2/GRE3*). Genes that encode

proteins involved in glutathione homeostasis were also expressed at the end of

fermentation, including *GEX1* that encodes a proton:glutathione antiporter (103, 104).

GEX1 is known to be induced during oxidative stress (103) and modulates formation of the

aromatic thiol 3-mercaptohexan-1-ol from its glutathionylated precursor in wines such as

Sauvignon Blanc (104). Conversely, an induction of a gene that encodes an enzyme that

cleaves glutathione (*GCG1*) was observed and may be involved in apoptotic signaling via

ROS accumulation (105).

Together, these gene expression patterns highlight how intertwined redox

homeostasis is with almost all core metabolic processes in *S. cerevisiae*, as most pathways

require oxidation or reduction by a pyridine nucleotide cofactor during at least one

reaction. For example, NAD+/H and NADP+/H participate in 740 and 887 biochemical

reactions through interactions with 433 and 462 enzymes, respectively (106). It is also well

documented that experimental perturbation of both NAD+/H and NADP+/H leads to

changes in aroma compounds in wine and other fermented beverages (107–110). The

observations presented herein conserved across many Pinot noir fermentations involving

genes engaged in redox balance and mitigation of oxidative stress via thiol-reductase

systems offers further evidence for the importance of these systems. These findings

provide motivation for future studies of these systems in the context of wine production,

which would include control measures to aid cellular control of redox and mitigate

oxidative cellular stress.

Stress-associated gene expression during fermentation

During fermentation, *S. cerevisiae* has to adapt to a continually changing stress

landscape. Macro- and micronutrients become limiting as ethanol concentrations increase

and, as discussed above, production of acetaldehyde and other metabolic processes

generate oxidative stress. To accommodate this dynamic environment, *S. cerevisiae* wine

strains express genes that overlap with, but are distinct from, the stress response of

laboratory strains. (7, 111). In accordance with previous studies (2, 3), we found partial

overlap between genes expressed in fermentation and those involved in the environmental

stress response (ESR) in laboratory strains. Specifically, 16 ESR genes were expressed at

the beginning of fermentation and 78 ESR genes at the end of fermentation. This matches

observations in synthetic must where stress genes were induced upon entry into stationary

phase (2). Stress-related genes expressed at the beginning of fermentation were enriched

for Gene Ontology pathways involving carbohydrate transmembrane transport (mannose,

fructose, glucose, hexose) and NADP regeneration **(Figure 3A)**, while stress-related genes

expressed at the end of fermentation were enriched for oxidation-reduction process,

generation of precursor metabolites and energy, energy reserve metabolic process, and

glycogen metabolic process **(Figure 3B).**

A recent study investigated fermentation of Riesling grape must at laboratory scale

without addition of oxygen (3). Using microarray analysis at five time points in

fermentation, the authors defined a fermentation stress response (FSR) as those genes that

are induced at any point in fermentation and do not return to baseline (3). The FSR is

differentiated from the ESR and common stress response because adaptation is not

observed over time through gene expression returning to pre-stress transcription levels (3,

7, 111). Of the 223 genes induced in the FSR, 84 were observed to be expressed in mid- or

late- fermentation. Of these 84 genes, 43 overlap with genes expressed in other stress

responses as defined in (3), including 16 with the ESR and 14 with the common stress

response. Of the 41 genes that overlap with the FSR, many were related to the challenging

nutrient environment in wine, including glucose limitation (*NRG1*, *SKS1*, *HXT6*, *VID24*),

nitrogen limitation (*MEP2*, *GAP1*, *PTR2*, *AVT4*, *VBA2*), vitamin limitation (*MCH5*, *VHR1*), and

stress caused by heat, salt, protein mis-folding, and cell wall defects (*GAC1*, *RPI1*, *JID1*,

PSR2). This suggests that multiple stress pathways are simultaneously activated by the

challenging environment that *S. cerevisiae* encounters in wine fermentation, which likely

defines the described fermentation stress response. Many genes identified in the FSR, and

expressed in this study during fermentation, remain uncharacterized (*YPR152C*, *YBR085C-

A*, *YDL024C*, *YDR042C*, *YMR244W*, *YLL056C*), offering gene targets of future investigation

related to adaptation to the fermentation and wine environment.

Polyol metabolism in late fermentation

Polyols, also called sugar alcohols, have recently been shown to be produced by non-

Saccharomyces yeasts and by fructophilic lactic acid bacteria such as *Lactobacillus kunkeei*

during fermentation (112, 113). Combined with other spoilage organism associated

metabolites, these compounds can have a significant impact on wine quality (114).

Mannitol is one such polyol and a non-preferred sugar that can be metabolized by *S.*

cerevisiae (115–117). In *S. cerevisiae*, transporters encoded by *HXT13* and *HXT15-17* were

found to facilitate mannitol and sorbitol transport (116). In our data, we observed

induction of the mannitol transporter *HXT13* in both vintages, along with the mannitol

dehydrogenase *MAN2*, which together indicate that mannitol may be present and

metabolized by *S. cerevisiae* at the end of fermentation (**Figure 4**). In line with this,

although eukaryotic transcriptional profiling via 3′ Tag-seq was performed (see methods),

L. kunkeei transcripts were detected in some fermentations, which is one potential source

of mannitol production. These data raises the possibility of mannitol consumption by *S.*

cerevisiae, demonstrating metabolic flexibility for carbon sources late in fermentation.

Notably, *L. kunkeei* can influence *S. cerevisiae* metabolism beyond expression of

genes for non-preferred carbon sources. Via production of acetic acid, and possibly other

compounds, *L. kunkeei* has been shown to induce the [GAR+] prion phenotype in *S.*

cerevisiae thereby shifting carbon metabolism away from hexoses (118, 119). Given that

the presence of *L. kunkeei* RNA was detected in the 2017 vintage, we tested for the

presence of the [GAR+] phenotype in the 2019 vintage via cell culture (119). We did not

detect the presence of the [GAR+] prion in any fermentation tested in the 2019 vintage.

While the absence of the [GAR+] phenotype in the 2019 vintage does not preclude its

presence in the 2017 vintage, consistent gene expression for mannitol transport and

degradation in both vintages suggests that *S. cerevisiae* may be metabolizing mannitol in

these Pinot noir fermentations due to the presence of *non-Saccharomyces* organisms,

including *L. kunkeei*.

Vintage-specific differences

From our data analyses, there are 717 genes and 375 genes differentially expressed

in the 2017 and 2019 vintages, respectively. The majority of these genes were members of

pathways enriched among all fermentations (**Figure 2C**, **Figure S1**-**Figure S4**). Using Gene

Ontology enrichment analysis, no molecular function, cellular compartment, or biological

process was enriched in either vintage that was not also enriched in both vintages. This

suggests these differences may be largely due to sequencing depth or variations in the gene

expression within these pathways and not differences in the overall biology of *S. cerevisiae*.

Still, signatures indicative of vintage-specific effects were observed, some of which may

impact the sensory attributes of wine. For example, glycerol is an important fermentation

byproduct that can contribute to the mouth feel of wine (120). *S. cerevisiae* uses glycerol

biosynthesis to generate NAD+, a required cofactor for glycolysis, when NAD+ levels are

not sufficiently replenished through fermentation (121). During glycerol biosynthesis,

enzymes encoded by *GPD1* and *GPD2* convert dihydroxyacetone phosphate into glycerol-3-

phosphate (122). Both *GPD1* and *GPD2* were expressed in early fermentation in the 2017

vintage, but not in the 2019 vintage. A second example involves genes encoding the

fluoride transporters Fex1 and Fex2, which were expressed in late fermentation across all

fermentations in the 2019 vintage. Fluoride is a toxic anion that *S. cerevisiae* exports via

two plasma membrane transporters to avoid cell damage (123), which in excess can cause

slow or stuck fermentation (124). Although fluoride is ubiquitous in terrestrial and aquatic

environments (123), application of the insecticide Cryolite, which contains fluoride, has

caused problematic fermentations in California vineyards (124). Currently, the reasons for

these gene vintage-specific expression patterns are not known.

Finally, it was observed that genes of currently unknown function were

differentially expressed in the two vintages assayed. Using a $\log_2$ fold change cut off of two,

14 genes in the 2017 vintage and seven genes in the 2019 vintage were of unknown

function. Across both vintages, more genes of unknown function were expressed in late

fermentation than in early fermentation (10 in 2017, 5 in 2019). Knowledge of the specific

pathways expressed in late fermentation due to the stressful, nutrient-limited conditions,

offers clues to the potential functions of these genes that could be explored in future work.

Conclusion

In this study, we present a gene expression analysis across 40 pilot-scale

fermentations of California Pinot noir wine using grapes from 10 vineyard sites and two

vintages. The fermentations were diverse with different kinetics, initial chemical

conditions, and microbial communities (56). Yet among this diversity, we detected a core

gene expression program by *S. cerevisiae* that is largely consistent with that observed at

laboratory scale (2–4). Given that there are many genes consistently expressed across

these Pinot noir fermentations from diverse vineyards, members of this core fermentation

gene program represent strong candidates for future study to impact wine outcomes,

e.g. through manipulating redox balance (107–110). Excitingly, this includes a large

number of genes with unknown function that through investigation may provide new

insights into the biology of *S. cerevisiae.*

The largest deviations from benchtop fermentations are likely attributed to

activities of non-*Saccharomyces* organisms, but more research is needed to understand

these complex ecological interactions and their impact on fermentation. The gene

expression signatures around oxygen presence and metabolic availability also warrants

further research, in particular into the role of the mitochondria in late fermentation (3, 6,

74). While we detected few vintage-specific differences between fermentations, we expect

there are vineyard-site specific deviations from the consistent patterns of gene expression

described herein. Given the variability in fermentation kinetics with respect to time of

sampling, new methods will likely be needed to resynchronize stages of fermentation to

enable cross-vineyard comparisons (4). Future work is also needed to extend these

observations to other grape varieties and *S. cerevisiae* wine strains, which will define both

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sampling, new methods will likely be needed to resynchronize stages of fermentation to

enable cross-vineyard comparisons (4). Future work is also needed to extend these

observations to other grape varieties and *S. cerevisiae* wine strains, which will define both

the shared and unique facets of the core gene expression program in *S. cerevisiae* linked to

these variables. With such information, we can address the impact of an industrial wine

fermentation environment on *S. cerevisiae* gene expression and define approaches that can

be used to manage commercial fermentation outcomes.

Methods

Grape preparation and fermentation

The wine making protocol used in this study was described previously (23, 48). The

grapes used in this study originated from 10 vineyards in six American Viticulture Areas in

California. All grapes were Pinot noir clone 667 rootstock 101-14. We harvested grapes at

approximately 24 Brix and transported the fruit to University of California, Davis Pilot

Winery for fermentation. We performed separate fermentations for grapes from each site,

with two fermentations per site, totaling of 20 fermentations per vintage (40 fermentations

total). After harvest, we separated the fruit into half-ton macrobins on harvest day and

added Inodose SO_2 to 40 ppm. We stored the bins in a 14°C cold room until destemming

and dividing of the fruit into temperature jacket-controlled tanks. We performed N_2

sparging of the tank headspace prior to fermentation and sealed tanks with a rubber

gasket. We cold soaked the must at 7°C for three days and adjusted TSO_2 to 40 ppm on the

second day. After three days, we increased the must temperature to 21°C and set a

programmed pump over timetable to hold the tank at a constant temperature. We

reconstituted *S. cerevisiae* RC212 with Superstart Rouge at 20 g/hL and inoculated the

must with 25 g/hL of yeast. At approximately 24 hours after inoculation, we adjusted

nitrogen content in the fermentations using DAP (target YAN – 35 mg/L – initial YAN)/2,

and Nutristart using 25 g/hL. We only adjusted nitrogen if target YAN was below 250

mg/L. Approximately 48 hours after fermentation, we permitted fermentation

temperatures to increase to 27°C and added DAP as previously described. Fermentations

ran to completion when Brix < 0. We took fermentation samples for Brix measurements

and RNA isolation at 16, 64, and 112 hours relative to inoculation. To ensure uniform

sampling, we performed a pumpover ten minutes prior to sampling each tank. For RNA

samples, we obtained 12mL of juice was obtained and centrifuged at 4000 RPM for 5

minutes. We discarded the supernatant and froze the pellet in liquid nitrogen. We stored

samples at -80°C until RNA extraction.

RNA extraction and sequencing

We thawed frozen yeast pellets on ice, resuspended in 5ml Nanopure water,

centrifuged at 2000g for 5min, and aspirated the supernatant. We extracted RNA using the

Quick RNA Fungal/Bacterial Miniprep kit including DNAsel column treatment (cat#R2014,

Zymo Research). We eluted samples in 30μL of molecular grade water and assessed for

concentration and quality via Nanodrop and RNA gel electrophoresis. We adjusted sample

concentrations to 200ng/μl and 20 μl sent for sequencing. We used 3´ Tag-seq single-end

sequencing (Lexogen QuantSeq) in both the 2017 and 2019 vintage, with the addition of

UMI barcodes in 2019. The University of California, Davis DNA Technologies Core

performed all library preparation and sequencing.

Differential expression analysis

We preprocessed samples according to manufacturer recommendations. First, we

hard-trimmed the first 12 base pairs from each read and removed Illumina TruSeq

adapters and poly A tails. Next, we used STAR to align our reads against *S. cerevisiae* S288C

genome (R64, GCF_000146045.2) with parameters --outFilterType BySJout --

outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --

outFilterMismatchNmax 999 --outFilterMismatchNoverLmax 0.6 --alignIntronMin 20 --

alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMattributes NH HI NM MD

--outSAMtype BAM SortedByCoordinate (125). For the 2019 vintage, we used UMI tools to

deduplicate alignments (126). We then quantified reads mapping to each open reading

frame using htseq count (127). We imported counts into R and filtered to mRNA

transcripts. To prepare for differential expression, we used the edgeR function

calcNormFactors with default parameters (128). We used limma for differential expression,

building a model using Brix values, preparing the data for linear modelling with the voom

function, and building a linear model for each gene with lmFit (129). We considered any

gene with an adjusted p value < 0.05 as significant. To combat batch effects from different

library preparation techniques used in the 2017 and 2019 vintages, we performed

differential expression separately on counts from each vintage. We took the union of

expressed and repressed genes between vintages, respectively, to generate the final set of

differentially expressed genes. We visualized expressed and repressed genes using

proteomaps (130), and visualized the intersection of differentially expressed genes

between vintages using the R package ComplexUpset

(https://github.com/krassowski/complex-upset).

We performed gene set enrichment analysis for genes that were expressed and repressed

in both vintages against the Gene Ontology (ont = "ALL") and Kyoto Encyclopedia of Genes

and Genomes (organism = "sce") databases using the R package clusterProfiler (131).

Detection of *Lactobacillus kunkeii* in RNA sequencing reads

3´ Tag-seq sequences the tail-end of transcripts that contain poly(A) tails. The

majority of transcripts with poly(A) tails are eukaryotic in origin, but given that bacteria

perform polyadenylation as a degradation signal (132), a very small subset of transcripts

may also originate from bacteria. We identified *Lactobacillus kunkeei* in RNA-seq reads

using sourmash gather (133, 134). Using all *L. kunkeei* genomes available in GenBank

(08/06/2019), we generated sourmash signatures for each using a k-mer size of 31 and a

scaled value of 100. We then used sourmash index to generate a database of *L. kunkeei*

genomes, and queried this database using signatures of each RNA-seq sample. To validate

findings from sourmash gather, we used BWA mem with default parameters to map a

subset of samples against the best matching *L. kunkeei* genome (135).

Culturing *Saccharomyces cerevisiae* for [GAR+] prion detection

To ascertain whether the [GAR+] prion state was detectable in wine fermentations,

we cultured yeast for the prion as performed in (119). We used yeast peptone-based

medium containing the designated carbon source, such as YPD (1% yeast extract, 2%

peptone, 2% agar, 2% glucose); YPG (1% yeast extract, 2% peptone, 2% agar, 2% glycerol)

or GGM (1% yeast extract, 2% peptone, 2% agar, 2% glycerol, 0.05% D-[+] glucosamine

hydrochloride). We inoculated yeast from fermentations into each well of a 96 well plate

containing 200µl liquid YPD + 34g/ml chloramphenicol, and then grew yeast at 30°C for 48

hours. We then pinned yeast to YPG or GGM plates and grew at 30°C for four days.

American Viticultural Area (AVA) map construction

We constructed the AVA map featured in **Figure 1** from the UC Davis Library AVA

project https://github.com/UCDavisLibrary/ava.

Data Availability

RNA sequencing data is available in the Sequence Read Archive under accession

number PRJNA680606. All analysis code is available at

github.com/montpetitlab/Reiter_et_al_2020_GEacrossBrix.

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1123

1124 **Table 1**: Genes differentially expressed throughout fermentation shared across vintages

1125 *Genes constitutively expressed in fermentation.*

Wine process	Gene	Expressed most highly	Gene product function
Glycolysis and fermentation	*HXT3*	late	Hexose transporter induced by both high and low glucose concentrations.
	PFK1, PFK2	*PFK2*: early	Phosphofructokinases that catalyze the first irreversible reaction specific to glycolysis, producing fructose-1,6-bisphosphate from fructose-6-phosphate.
	ADH1-ADH5	*ADH2-ADH4*: early *ADH5*: late	Alcohol dehydrogenase isoenzymes. Dominant fermentative alcohol is *ADH1* (136). Responsible for reoxidation of NADH to NAD+ which is a required cofactor in the metabolism of glyceraldehyde-3-phosphate in glycolysis. *ADH2* is a non-dominant isoenzyme of alcohol dehydrogenase. Typically repressed by glucose and converts ethanol to acetaldehyde (136). Overexpressed in some wine strains (137).

1126

1127 *Genes expressed higher in early fermentation in both the 2017 and 2019 vintages.*

Wine process	Gene	Gene product function
Glycolysis and fermentation	*HXT1*	Low affinity hexose transporter.
	HXK2	Hexokinase that phosphorylates glucose in the first irreversible step leading to glycolysis.
	PFK1, PFK2	Phosphofructokinases that catalyze the first irreversible reaction specific to glycolysis, producing fructose-1,6-bisphosphate from fructose-6-phosphate
Acetate metabolism	*ALD4-6*	Aldehyde dehydrogenase isoenzymes that produce acetate as a byproduct when acetaldehyde is metabolized. *ALD6* encodes the main isoenzyme responsible for acetate production in wine (96). Aldehyde dehydrogenase isoenzymes *ALD4* and *ALD5* are expressed when ethanol is the carbon source and are not typically associated with wine fermentation. Acetate contributes the majority of volatile acidity associated with negative organoleptic properties in wine (138).
	PDR12	Plasma membrane ABC transporter. Required for development of resistance to weak organic acids, including acetate (139).
Cell cycle	*BAT1*	Expressed in logarithmic phase (140).
	CWP1	Expressed in the S/G2 phase of cell cycle (140).
Nitrogen metabolism	*GNP1*	High-affinity glutamine permease that also transports leucine, serine, threonine, cysteine, methionine and asparagine.
	MUP1	High-affinity methionine permease that is also involved in cysteine transport.

	CAR1, CAR2	Involved in arginine catabolism. Arginine is the most abundant amino acid in grape must after proline (66) and is used in protein synthesis during fermentation (141).
	YPQ1	Vacuolar transporter for arginine and lysine. Unused arginine is stored in the vacuole for later use (141).
Ehrlich pathway	BAT1, ARO8	Catalyzes transamination of amino acids, the product of which cannot be redirected to central carbon metabolism and so is excreted as fusel acid or fusel alcohol (142). Overexpression of BAT1 increases the concentration of isoamyl alcohol, its acetate ester, and isobutanol in wine (142).
	PDC1	Catalyzes alpha keto decarboxylation.
Glycerol biosynthesis	GPP1	Cleaves phosphate from glycerol-3-phosphate.

1128

1129   *Genes expressed higher in late fermentation in both the 2017 and 2019 vintages.*

Wine process	Gene	Gene product function
Nitrogen limitation	GAT1, DAL80	Transcriptional activator (GAT1) and repressor (DAL80) of genes under nitrogen catabolite repression. Expression is inversely correlated, and the detection of both genes as induced in late fermentation likely indicates tight transcriptional regulation of nitrogen metabolism.
	DAL2-DAL5, DAL80, DAL82	Catalyze allantoin degradation and expression is under nitrogen catabolite repression.
	MEP2	Ammonia permease and expression is under nitrogen catabolite repression.
	GAP1	Amino acid permease.
	PTR2	Peptide permease.
	AVT3, AVT4	Vacuolar amino acid exporters that mobilize internal nitrogen stores for cell maintenance during stationary phase. Expression is under nitrogen catabolite repression.
ubiquitin-mediated selective protein degradation	RPN4	Transcription factor that induces expression of proteasome genes.
	TMC1	Effector of proteotoxic stress induced by nitrogen limitation, weak acid, and misfolded proteins. Target of RPN4.
	UBC8, VID24	Negative regulators of fructose-1,6-bisphosphate through ubiquitination (UBC8) and vacuolar targeting (VID24).
	UBC1, UBC5, UBC7, UBC13	Ubiquitin conjugating enzymes.
	UBI4, CUZ1	Involved in the ubiquitin-proteasome pathway.

Autophagy	*ATG2, ATG4, ATG7-ATG12, ATG14, ATG32, ATG40*	Proteins involved in autophagy. Autophagy is a key response to nutritional limitation that allows cells to maintain homeostasis (143). Nitrogen starvation leads to the largest autophagic response in yeast.
Erlich pathway	*GRE2*	Final step of pathway where fusel aldehydes are oxidized or reduced into fusel acids or alcohols (142).
Carbon limitation	*SNF3*	Plasma membrane low glucose sensor involved in regulating glucose transport
	SKS1	Serine/threonine kinase involved in the adaptation to low glucose via *SNF3*-independent signaling
	PGM2	Phosphoglucomutase. Catalyzes a key step in hexose metabolism. Induced in response to glucose limitation and ethanol stress (144).
	HXK1	Hexokinase that phosphorylates glucose or fructose in the first irreversible step leading to glycolysis. Under glucose-induced repression.
	HXT4, HXT6	Hexose transporters required at the end of alcoholic fermentation.
Trehalose and glycogen	*TSL1, NTH1, ATH1*	Involved in trehalose synthesis (*TSL1*) and degradation (*NTH1, ATH1*). Trehalose acts a a storage carbohydrate for cell maintenance in non-growth conditions (145, 146), bolsters membrane integrity by displacing ethanol (146), and protects proteins from denaturation (147). Trehalose recycling is an important component of stress response (148).
	GLG1, GSY1, GSY2, IGD1, GPH1, SGA1, GAC1, GIP1, YPI1, PIG2, GLC8	Involved in glycogen accumulation (*GLG1, GSY1, GSY2, IGD1*), degradation (*GPH1, SGA1*), and metabolism (*GAC1, GIP1, YPI1, PIG2, GLC8*). Glycogen accumulates during nutrient abundance and is metabolized during stationary phase and nutrient deprivation (145). Glycogen recycling is an important component of stress response (148).
Cell wall integrity	*PIR3, SED1, SLT2*	Target genes of the cell wall response to ethanol. *PIR3* is required for cell wall stability and induced in part by *SLT2*. *SED1* is a stress-induced cell wall structural protein (*SED1*) (149).
	PKH2, YPS1, PST1, KRE1	General cell wall integrity response.

1130

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Figure 1: California vineyard locations and fermentation patterns. A). Map displaying the six American Viticulture Areas (AVAs) in which the 10 study vineyards are located. B.) Fermentation curves reflecting the change in Brix over fermentation. Brix is a measure of total soluble solids that is used as a proxy for sugar concentration in grapes, grape must, and wine. C). Brix at time of sampling for each RNA-seq sample relative to inoculation. While samples were taken at the same absolute time, fermentations proceeded at different rates leading to different Brix values in each fermentation.

1139

Figure 2: **Transcriptome remodeling in fermentation is consistent across fermentations and vintages. A.)** *Genes expressed in early (*HXT1*), late (*HXT4*), and constitutively (*ADH1*) in fermentation.* **B.)** *Upset plot showing the intersection of genes that are significantly expressed at the beginning and end of fermentation in each vintage using a log$_2$ fold change cut off of 1. The majority of genes are consistently expressed across fermentations and vintages.* **C.)** *Proteomaps depicting Gene Ontologies (left) and genes (right) that are expressed in early (top) and late (bottom) fermentation. The size of individual genes reflects the associated log$_2$ fold change value. Note that for presentation purposes not all genes that are significantly expressed are depicted. See Table S1 for a complete list of genes.*

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Figure 3. Pathways enriched among genes differentially expressed across fermentation that are shared with the environmental stress response (ESR). *A.) Of 16 genes that overlap with the ESR and were expressed in early fermentation, pathways related to carbohydrate metabolism were enriched. **B.)** Of 78 genes that overlap with the ESR and were expressed in later fermentation, pathways related to oxidation-reduction and carbohydrate metabolism were enriched.*

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1157 ***Figure 4. Normalized log gene expression counts for genes involved in mannitol***
1158 ***transport and degradation.*** *HXT13 and MAN2 expression is graphed in the 2017 (**A, B**) and*
1159 *2019 (**C, D**) vintages. MAN2 and HXT13 were the top most expressed genes at the end of*
1160 *fermentation in 2019, and only fell behind HXT4 in the 2017 vintage. Grey lines indicate a*
1161 *linear model fit to normalized counts.*

1162

1163 **Figure S1. Significantly enriched Gene Ontology (GO) pathways in early fermentation**.
1164 *Pathways from GO categories molecular function, cellular component, and biological process*
1165 *are represented. Significant pathways are defined as p < 0.05 after Bonferroni p value*
1166 *correction.*

1167

1168 **Figure S2. Significantly enriched KEGG pathways in early fermentation.** *Significant*
1169 *pathways are defined as p < 0.05 after Bonferroni p value correction.*

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Figure S3. Significantly enriched Gene Ontology (GO) pathways in late fermentation.
Pathways from GO categories molecular function, cellular component, and biological process
are represented. Significant pathways are defined as p < 0.05 after Bonferroni p value
correction.

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Figure S4. _Significantly enriched KEGG pathways in late fermentation._ _Significant_

pathways are defined as p < 0.05 after Bonferroni p value correction.