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Original Article

Genome-wide effect of non-optimal temperatures under anaerobic conditions on gene expression in *Saccharomyces cerevisiae*

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ABSTRACT

Understanding of thermal adaptation mechanisms in yeast is crucial to develop better-adapted strains to industrial processes, providing more economical and sustainable products. We have analyzed the transcriptomic responses of three *Saccharomyces cerevisiae* strains, a commercial wine strain, ADY5, a laboratory strain, CEN.PK113-7D and a commercial bioethanol strain, Ethanol Red, grown at non-optimal temperatures under anaerobic chemostat conditions. Transcriptomic analysis of the three strains revealed a huge complexity of cellular mechanisms and responses. Overall, cold exerted a stronger transcriptional response in the three strains comparing with heat conditions, with a higher number of down-regulating genes than of up-regulating genes regardless the strain analyzed. The comparison of the transcriptome at both sub- and supra-optimal temperatures showed the presence of common genes up- or down-regulated in both conditions, but also the presence of common genes up- or down-regulated in the three studied strains. More specifically, we have identified and validated three up-regulated genes at sub-optimal temperature in the three strains, *OPI3*, *EFM6* and *YOL014W*. Finally, the comparison of the transcriptomic data with a previous proteomic study with the same strains revealed a good correlation between gene activity and protein abundance, mainly at low temperature. Our work provides a global insight into the specific mechanisms involved in temperature adaptation regarding both transcriptome and proteome, which can be a step forward in the comprehension and improvement of yeast thermotolerance.

1. Introduction

The yeast *Saccharomyces cerevisiae* has been widely used as a microbial cell factory to produce a wide variety of by-products, going from fermented food such as beer, wine, cider and bread to biofuels and pharmaceuticals [1,2] and has a central role on the development of biorefineries [3]. Due to the severe stresses accounted during industrial fermentations, yeast cells have to adapt to multiple harsh conditions [4]. Among them, temperature is one of the most relevant factors influencing yeast growth and fermentation performance, representing a major economic concern in the biotechnology sector. Industry invest large amounts of energy in controlling the temperature of the fermentation processes, sacrificing, in many cases, the optimum growth temperature [5,6]. In addition, in lignocellulosic biorefineries the process is favored if supra-optimal temperatures, close to the optimum temperature of the hydrolytic enzymes, can be used. Hence, unraveling the mechanisms that underlie yeast adaptation at high and low temperatures would allow the generation of better-adapted yeast to non-optimal temperatures, which would benefit industries in terms of energy costs and productivity.

Several genes have been related to high temperature tolerance in *S. cerevisiae*, mainly involved in membrane remodeling such as *ERG3* [7], a C-5 sterol desaturase; *ERG13* [8], a protein involved in early ergosterol biosynthesis; chaperones like *HSP104* and *HSP12* [9];

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trehalose and glycogen genes *TPS1*, *TPS2*, *NTH1* [10] and *GSY1* [8]; cAMP-PKA signaling pathway [11] or genes of RNA processing such as *PRP42* and *SMD2* [12]. The up-regulation of *RSP5*, a ubiquitin ligase, has also been proved to increase thermotolerance in yeast [13].

Likewise, low temperature adaptation arise a wide range of biochemical and physiological effects in yeast cells: poorly efficient protein translation; low fluidity membrane; changes in lipid composition; slow protein folding; stabilization of mRNA secondary structures and reduced enzymatic activities [14–21]. Therefore, specific genetic mechanisms are triggered in order to counteract the deleterious effect of cold environment such as *MUP1*, a high-affinity methionine permease involved in cysteine uptake and *AHP1*, a thiol-specific peroxiredoxin that reduces hydroperoxides [15]; the lipid genes *DPL1*, *LCB3*, *OLE1* and *PSD1* [14,22]*; SNU66* and *PAP2*, related to ribosome biogenesis [16] or the membrane gene *FPK1*, that maintain plasma membrane asymmetry by flipping specific phospholipids from the extracellular to the cytosolic leaflet [20].

In recent years high throughput RNA sequencing (RNA-seq) has become the method of choice for analyzing changes in gene expression between cells grown in different conditions [23–25]. In this sense, several attempts have been made to elucidate the transcriptional response of *S. cerevisiae* to temperature changes. These studies have been mainly focused on temperature shock [7,19,21,26–31]. However, the type and magnitude of the response is very dependent on the length of exposure to stressful conditions. Temperatures shocks are likely to trigger a fast and dynamic response (adaptation), while longer exposures to non-lethal stimuli leads to acclimation; i.e., establishment of a physiological state in which regulatory mechanisms, like gene expression, fully adapt to suboptimal environmental conditions [18].

With the aim of elucidating the molecular mechanisms governing yeast response to sub- and-supra optimal temperatures under anaerobic conditions, we applied RNA-seq to characterize the transcriptomic remodeling of three phenotypically divergent *S. cerevisiae* strains [32]: Ethanol Red (bioethanol strain), ADY5 (wine strain) and CEN.PK113-7D (laboratory reference strain). Those strains were selected due to its thermotolerance. ADY5 is better-adapted to grow at low temperatures, Ethanol Red is able to survive at higher temperatures while the lab strain was used as a control [32]. To avoid the interferences due to the specific growth rates of each strain and temperature [32,33], the strains were cultured at 12, 30 and 39 °C, in anaerobic chemostat cultures, at a fixed specific growth rate of 0.03 h^{-1}, enabling an accurate study of temperature responsive genes. Using independent culture replicates, we have identified differential expressed genes (DEG) for the three strains at low and high temperature. Beyond unraveling the most dominant mechanisms underlying temperature adaptation, the use of three strains with different thermotolerance and isolated from different niches enabled us to identify strain-dependent and temperature-dependent physiological differences, providing the necessary knowledge for the further production of better adapted to non-optimal temperature yeasts.

2. Results and discussion

2.1. Overview of the genomes and transcriptional changes to non-optimal temperatures

We investigated the genetic basis of non-optimal temperature adaptation in three *S.cerevisiae* strains from different ecological niches, characterized in a previous study [32], as displaying differences in their thermotolerance. The genomes of these three strains were sequenced and compared with that of reference strain S288c. Fig. S1 shows the changes in read-depth between chromosomes in the three strains. As it can be observed, the three strains are free from aneuploidies. While CEN.PK113-7D is a haploid strain, both industrial strains ADY5 and Ethanol Red are diploid. In addition, as small duplication/deletion in a genome can sometimes lead to phenotypic differences [34], we investigated the small duplication/deletion events in our strains (Table S1). The comparison with the reference strain yielded 112, 107 and 54 strain specific indels longer than 1000 bp, in the ADY5, Ethanol Red and CEN.PK113-7D, respectively. As expected, subtelomeric families showed drastic copy number variation comparing with the reference and also among strains [35,36]. Regarding the industrial strains, it can be seen the increase in the copy number of *SGE1* and *ARR1* genes in both strains. These genes have already been related to low temperature adaptation [20]. In addition, ADY5 also present more copies of the genes *SAM3* and *SAM4* while Ethanol Red had an increase in *AQY1*, *HPA2* and *OPT2*, which are also described to be involved in temperature adaptation [17,20].

Based on the raw sequence data, we identified 23,367, 5389 and 44,024 mutations in ADY5, CEN.PK113-7D and Ethanol Red, respectively, in comparison to the reference strain (Fig. S2 and Table S1). As expected, the lab strain presented a smaller number of SNVs with the reference (lab strain) comparing with the industrial strains. Values ranging from 31 to 38% of the SNVs located in coding regions of the genome were non-synonymous and could potentially affect the phenotype (Table S1).

The heat and cold conditions applied in this study are sub-optimal growth conditions and represent similar but inverse environmental questions relative to ambient regulation conditions. In this sense, we can differentiate both the extent of the changes in gene expression levels as a function of the temperature and the specific changes in the gene expression profiles in yeast strains from different ecological niches.

To obtain an overview of the samples distribution regarding strains and the three temperature conditions variability, a Principal Component Analysis (PCA) was carried out (Fig. 1, Table S2). The PCA score plot of the first two principal components comprised the 59% of the total variance. The use of these components in a 2D representation allowed for the effective separation of the samples based on the strain and growth temperature. PC1 was responsible of the 37% of the variance and perfectly separated the three different strains, with a clear differentiation of the laboratory strain CEN.PK113-7D from the two industrial strains under study. PC2 accounted for 22% of the total variance and separated the samples according to temperature. In addition, good

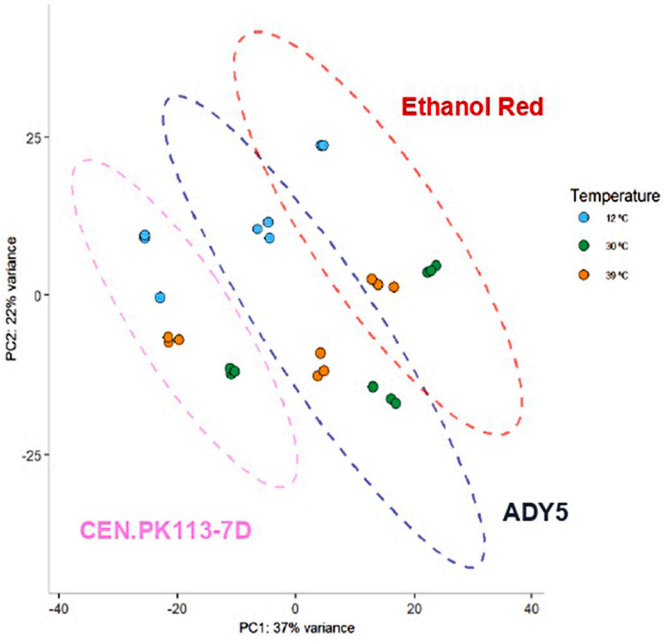

Fig. 1. Principal component analysis (PCA) of differential gene expression for the three *Saccharomyces cerevisiae* strains assayed (ADY5, CEN.PK117-3D and Ethanol Red) in the three different growth temperatures (12, 30 and 39 °C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

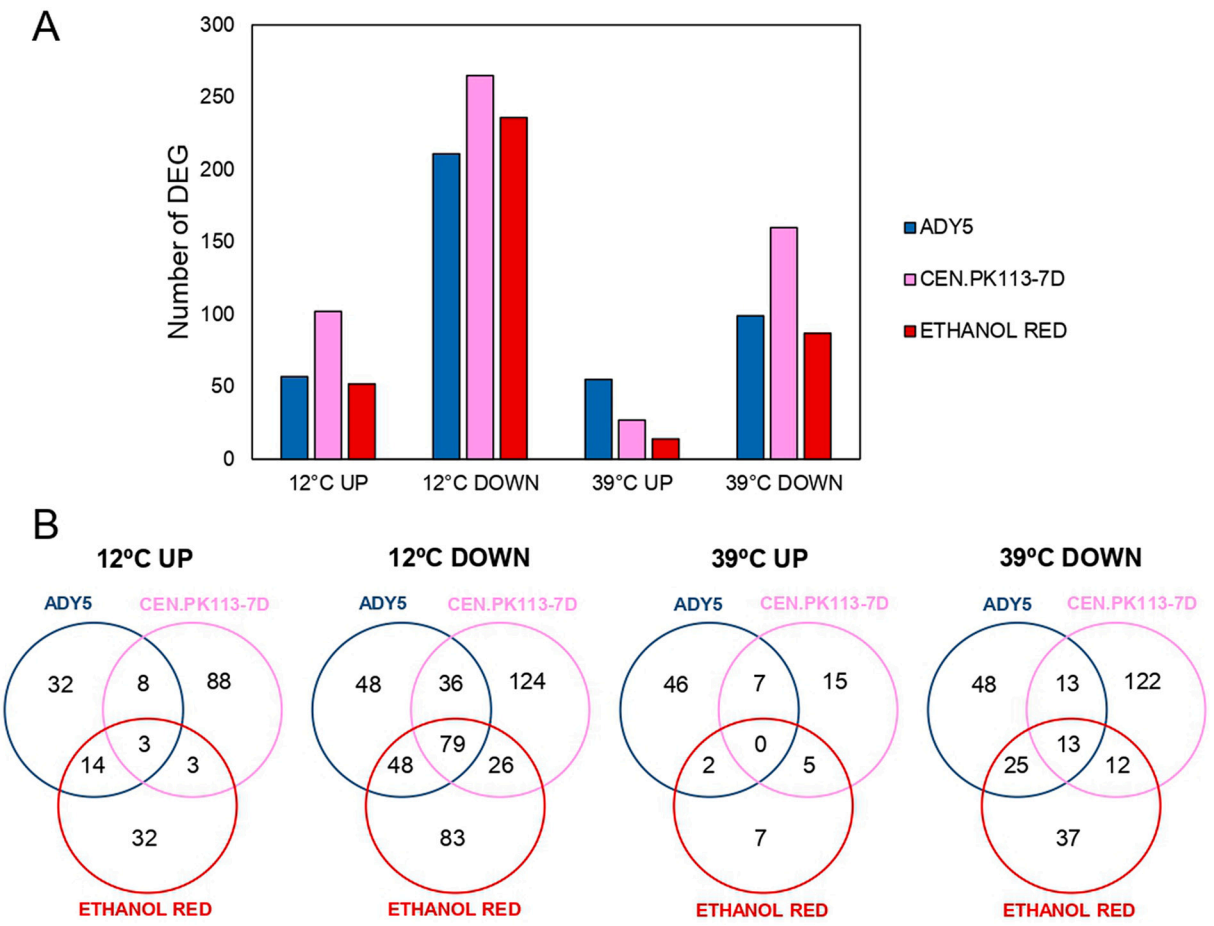

Fig. 2. (A) Number of differential expressed genes (DEG) in the three *Saccharomyces cerevisiae* strains assayed (ADY5, CEN.PK117-3D and Ethanol Red) at low and high temperature (12 and 39 °C). (B) Venn diagrams to show the overlap of the DEGs in the three strains at low and high temperature (12 and 39 °C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlation among the three replicates was verified.

Temperature effect for each strain separately was analyzed setting 30 °C as the reference condition. Interestingly, low temperature exerted a stronger transcriptional response in the three strains reflected by the higher number of differential (log2 fold change$\leq \pm 1.5$, p-value≤ 0.01) expressed genes (DEG) compared with heat condition, indicating greater remodeling of the transcriptome (Fig. 2A, Table S3). However, higher target gene expression does not necessarily mean that the corresponding protein is also abundant or indeed active in the cell [37–39]. Nonetheless, our results were concordant also with a greater proteome remodeling at low temperature described for the three studied strains [8], as it is analyzed below.

More specifically, in ADY5 strain, the number of DEG at 12 °C was 268, whereas at 39 °C it was 154; in the case of CEN.PK113-7D, we identified 367 DEG at low temperature and 187 at high temperature; and in Ethanol Red, 288 DEG at low temperature, and only 101 at high temperature (Fig. 2A and Fig. S3). From the point of view of temperature adaptation of the three strains, this is somewhat striking. One might expect that the strain best adapted to low temperature (ADY5) would need a deeper gene expression rewiring to high temperature, and thus the highest number of DEG in order to reach a suitable physiological state. Similarly, the high temperature adapted strain Ethanol Red would need a high transcriptomic reprogramming at low temperature. However, what we saw here is an even highest number of DEG found in the mesophilic lab strain CEN.PK113-7D. That would be in line with the existence of a general transcriptomic response to deal with stressful temperature conditions, so any strain adapted to non-optimal temperature, regardless it is low or high, would be more prepared to also adjust its gene expression to the opposite temperature range than a strain that is not adapted to non-optimal temperatures at all [40–43]. Nonetheless, it should be also taken into account that the industrial strains are more prone to cope with stress situations [44–46].

Moreover, regardless the growth temperature, the number of the down-regulated genes was notably higher than the up-regulated genes in the three strains (Fig. 2A and S2), highlighting that probably is necessary to turn off several genes and cellular processes in order to cope with these harsh conditions. Lee et al. [38] proposed a model in which explained transcriptional reduction during environmental stress as a molecular mechanism to avoid competition with translation factors, particularly initiation factors that regulate translation in response to stress, but also to prevent competition for translating ribosomes. Nearly 90% of ribosomes in growing cells are actively translating proteins, leaving little capacity to synthesize new proteins during adversity [31,47]. On the other hand, yeast cells, when exposed to stress, can enter in a protective state in which cell division, growth, and metabolism are down-regulated [48–51]. In all three scenarios, the cellular changes may increase the survival of the cells.

2.2. Global transcriptomic response to non-optimal temperatures

The capacity of yeast cells to remodel and adapt to non-optimal temperatures starts with changes in gene expression [52]. To examine these changes, we studied the DEG of the three strains at 12 and 39 °C, compared to the respective control temperature (30 °C) (Table S1). Venn diagrams were performed to identify common expression changes in the three strains at 12 and 39 °C (Fig. 2B). Only three genes were commonly

up-regulated at low temperature, *OPI3*, *EFM6* and *YOL014W* while 79 genes were commonly down-regulated in the three strains (Table S4), mainly related with transmembrane transport of different compounds such ions, carbohydrates and amino acids. Regarding heat, 13 genes were down-regulated in the three strains involved in oxido-reduction reaction and as a constituent of the cell wall. We did not detect any common gene to the three strains up-regulated at 39 °C.

In order to validate the role of some of these genes in the global thermal response, deletion mutants were constructed for the three genes commonly up-regulated at 12 °C (*OPI3*, *EFM6*, and *YOL014W*). Fig. 3A shows the specific growth rates of the mutants and WT strains at low temperature in the same medium used for the chemostat cultivations. It is noteworthy to remark that ADY5 strain, selected by its cryotolerance, presented an impairment in their growth at low temperature when any of the three selected genes was deleted but mainly with *YOL014W* and *OPI3*.

More in detail, *OPI3* mutants provoked an impairment in the growth rate of both industrial strains at low temperature. *OPI3* encodes for a methylene-fatty-acyl-phospholipid synthase, which catalyzes the last two steps in phosphatidylcholine biosynthesis. Interestingly, the deletion of this gene has previously been related with cold sensitivity [14,22]. Phosphatidylcholine (PC), the major phospholipid (at least 30% of total PLs), is synthesized de novo from another PL, phosphatidylethanolamine (PE), in three SAM-consuming methyltransferase reactions [53]. We hypothesize that *OPI3* is strongly up-regulated in cold because the demand of PC enhances in order to increase the membrane fluidity at low temperature [54]. Previous work has also shown up-regulation in the sulfur assimilation pathway at low temperature [17]. This pathway incorporates extracellular sulfate into several key sulfur-containing compounds such as S-adenosylmethionine (SAM), also involved in the PC biosynthesis.

YOL014W is a putative protein of 124 amino acids with unknown function. Its deletion causes a decrease in the growth of ADY5. Due to the lack of information about this protein and its huge impact on yeast growth at low temperature, further studies are needed in order to elucidate its possible function in the cell. Curiously, when we compare the sequence of this gene in the three strains, it can be observed that CEN.PK113-7D and Ethanol Red presents a 21 bp deletion in this gene, which could be the reason why ADY5 mutant is the unique strain with a defective phenotype at cold temperature. This, together with the fact that it presents several SNVs, indicates high variability of *YOL014W* within the species, which could be a symptom of pseudogenization [55]. Indeed, one of these SNVs provokes a truncated protein in the case of CEN·P113-7D due to a premature stop codon (Fig. 3B). All these data point out to ADY5 being the only strain out of the three with a version of the gene that is useful for low temperature tolerance, or even functional at all. In addition, when we study the conservation degree of the this ADY5 gene within *Saccharomyces* genus by using BLAST, we observed a percentage of identity ranging from 85 to 100% in *S.cerevisiae* and some of its hybrids while the identity decreases from 83 to 71% in the other species of the *Saccharomyces* genus, highlighting the great variability of this gene.

On the other side, *EFM6*, a putative S-adenosylmethionine-dependent lysine methyltransferase, which modifies Lys-390 in translational elongation factor eEF1A [56], impaired ADY5 and CEN.PK113-7D growth. However, the effect of this modification of Lys-390 is not yet clear. eEF1A (encoded by the *TEF1* and *TEF2* genes) is an aminoacyl-tRNA transferase needed during protein translation. However, eEF1A is a moonlighting protein which, beyond its role in translation elongation, also acts in several cellular processes, including nuclear export and F-actin remodeling [57]. Moreover, *EFM6* may potentially methylate other unknown proteins, which could be acting over cellular processes involved in low temperature adaptation. Although the gene sequence of the three strains were identical, ADY5 presents one upstream SNV located in the promotor region of the gene, which could potentially affect the phenotype of this strain (Table S1).

The complete set of constructed mutants were also tested at 30 °C comparing with the WT strains (Fig. S4). None of the mutant strains showed an impaired growth at optimum temperature, with the exception of *OPI3*, whose deletion also affected the growth rate of the three

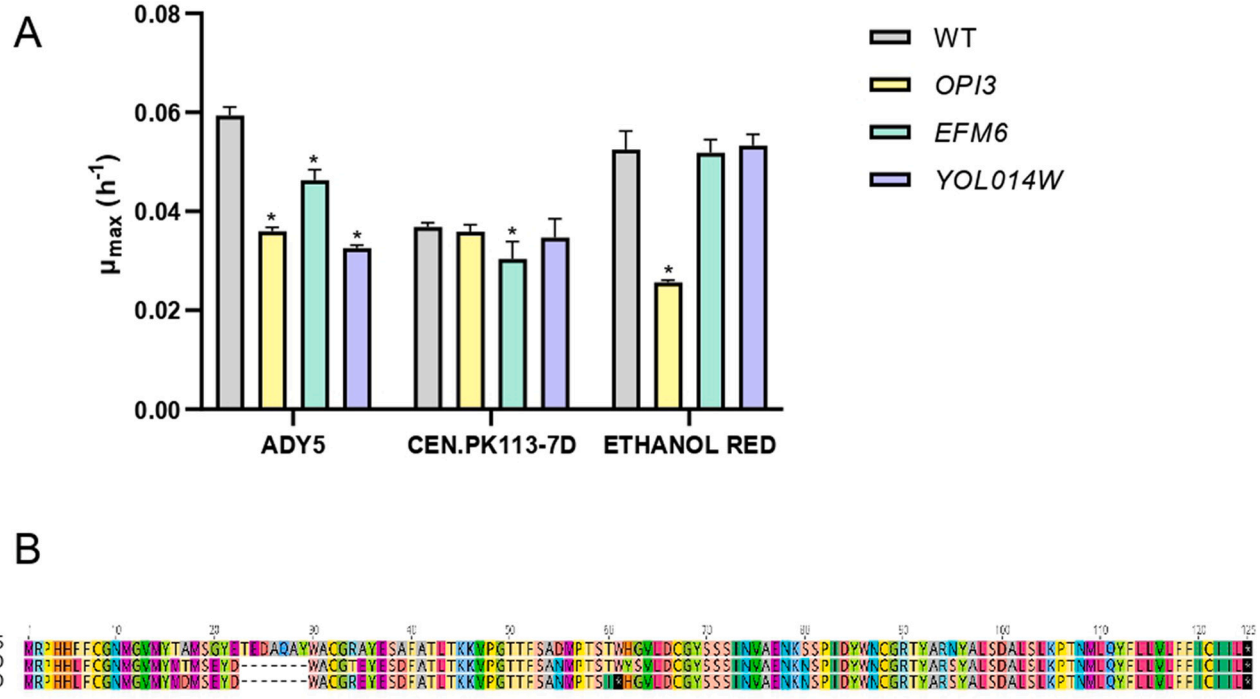

Fig. 3. Phenotypic and genomic analysis of the validated genes. (A) Growth rate (h^{-1}) of the constucted deletion mutants for *OPI3*, *YOL014W* and *EFM6* in ADY5, CEN.PK117-3D and Ethanol Red at12 °C. *Significant differences compared with the control ($p \leq 0.05$). (B) YOL014Wp multiple alignment: *YOL014W* coding sequences of each of the strains were obtained from the RNAseq alignments against the RNAseq reference. The translated protein sequences were aligned with ClustalW. (*) indicates a stop codon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strains. Although this decrease was much smaller than the phenotype observed in cold as it was previously described [14].

In addition, gene ontology analysis was carried out to determine the main functional categories arising from the up- and down- regulated genes in the three strains. A total of 94 and 69 enriched GO terms among the three strains (Fig. 4 and Table S4) at 12 and 39 °C, respectively, were identified revealing the complexity of the cell's response to temperature changes. Fig. 4 shows a heatmap of the GO terms obtained at 12 °C as a function of their *p*-values (the darker colour, the lowest *p*-value) in the three strains. Curiously but not unexpected, when samples were clustered, three different subgroups can be observed, the group 1 and the group 2 integrated by the down-regulated and up-regulated categories, respectively, in the two industrial strains, ADY5 and Ethanol Red and, the group 3 with both up- and down-regulated categories of the lab strain. Both industrial strains were selected to be adapted at low (ADY5) or high (Ethanol Red) temperature and their adaptive responses are similar and more focused compared to the response of CEN.PK strain, that is very scattered and less specialized. It is known that lab strains

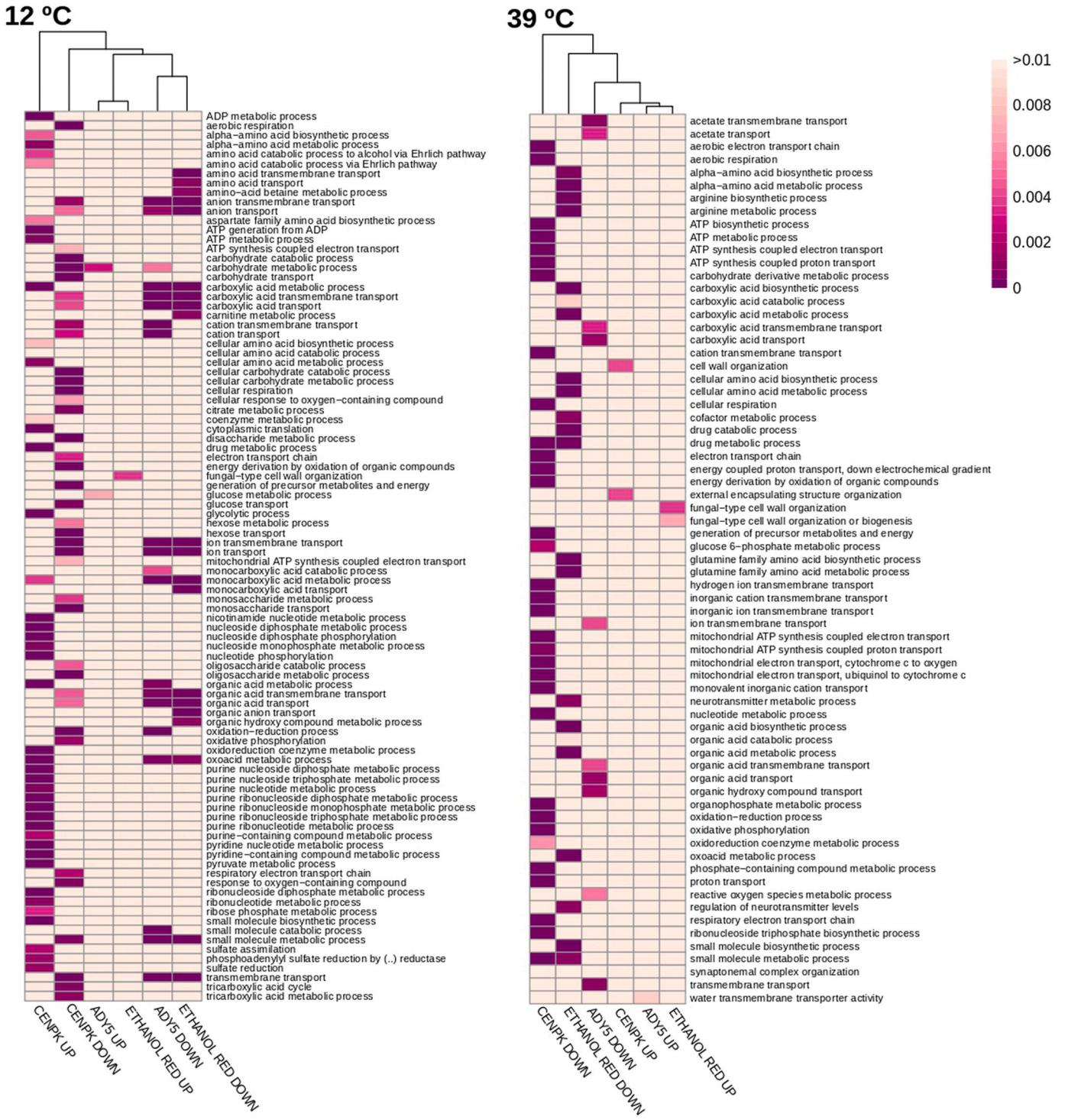

Fig. 4. Heatmap of sample clustering according to the *p*-values (the darker the colour, the lowest *p*-value) of the functional categories obtained at 12 °C and 39 °C in the three strains.

differ, both genomic and phenotypically, from industrial strains, which are more robust and adapted to harsh environments [58,59]. More in detail, ADY5 and Ethanol Red showed a down-regulation of genes belonging to functional categories related with amino acid, ion and organic acids transport and the metabolism of small molecules and carboxylic acid. Regarding the overexpressed genes in ADY5 at low temperature they were all grouped in carbohydrate and glucose metabolism while the focus of Ethanol Red transcriptional response was the cell wall organization, mainly mannoprotein encoding genes, which belong to the *DAN/TIR* family and the subtelomeric *PAU* family. Those cell wall mannoprotein genes have been widely linked to low temperature response [60–62].

In contrast, the lab strain presented 42 and 40 functional categories, for its up- and down-regulated genes, respectively, at low temperature (Fig. 4). Besides this huge response created by CEN.PK113-7D to deal with cold stress, overexpressed genes can be grouped into ATP production and glycolytic process, translation, purine and pyrimidine metabolism and sulfate-related process. The up-regulation of sulfate-related process has been previously reported al low temperature in order to increase the levels of SAM required for the synthesis of phosphatidylcholine (PC), the major phospholipid in the membranes and key in cold adaptation [17,63–65].

Regarding high temperature (Fig. 4), a clear separation can be observed between up- and down-regulated categories regardless the analyzed strain. Nevertheless, downregulated genes resulted in many and diverse significant functional categories, while in the case of upregulated genes only a few of functional categories arose. In the three strains, the up-regulated genes at high temperature were related with membrane structures, to counteract the increase in fluidity that undergo the cellular membranes, and processes such as sporulation, consistent with the idea that cells prepare for dormancy to ensure survival [52,66]. Transmembrane transport of water was significant in ADY5 strain in order to balance the loss of intracellular water due to the membrane fluidification. In most organisms, water crosses the plasma membrane by two parallel pathways: (1) the lipid bilayer with a high activation energy (Ea) for transport and lower osmotic permeability coefficients (Pf), and (2) the channel pathway (through aquaporins), with a low Ea and higher Pf values [67].

Regarding the downregulation response at high temperature, CEN.PK113-7D down- was, again, the condition that presented more differences compared with the others, with functional terms very related to aerobic respiration. Ethanol red, however, presented a response mostly consistent on down-regulation of the biosynthesis of alpha-amino acids, carboxylic acids, cofactors and different small molecules. This response was similar with that obtained for the down-regulated set of genes at 12 °C in Ethanol Red. The synthesis of these products is ATP-dependent, thus correlating with our previous results that indicated an optimized system of cellular energy maintenance in this strain in comparison to the other strains at both high and low temperature [32]. Finally, ADY5 focused its response on the down-regulation of membrane transporters. Several transporters act by the so-called mechanism proton-coupled symport [68]. As above mentioned, high temperature decreases the cytoplasmic pH as consequence of excessive membrane fluidity [69] and the downregulation of the membrane transports may be a mechanism to deal with this increase in the proton levels.

Table 1
Summary of nodal transcriptions factors in gene interaction networks obtained with *PheNetic*.

	12 °C	39 °C
ADY5	Pub1p, Ste12p, Cin5p, Yrm1p, Spt23p,Nrg1p	Ste12p, Cin5p, Spt23p, Mga1p
Ethanol Red	Pub1p, Ste12p, Spt23p, Abf1p	Pub1p, Ste12p, Spt23p, Msn2p
CEN.PK113-7D	Pub1p, Ste12p, Cin5p, Spt23p, Msn2p, Mip6	Ste12p, Yrm1p,Spt23p, Abf1p

DEGs for every temperature and strain were analyzed with *PheNetic* web tool (Table 1 and Fig. S5), which uses publicly available interactomics data to create networks from a given expression dataset, in order to identify master regulators relevant for the sample [70]. Table 1 shows the main transcription factors governing each of datasets. At a glance, it can be observed Pub1p, Ste12p, Cin5p and Spt23p as the most common factors for all the conditions. Pub1p is an RNA-binding protein involved in post-transcriptional regulation of the expression of several genes, stress granule formation, and maintenance of the tubulin cytoskeleton [71]. Ste12p activates genes involved in mating or pseudohyphal/invasive growth pathways [72] and it has been also described as a master regulator at low temperature in *S.cerevisiae* [30]. Cin5p is a member of the Yap protein family participating in several pathways including diverse stress responses [73]. Spt23p is a membrane protein that regulates *OLE1* transcription [74]. *OLE1*, the only desaturase of *S. cerevisiae*, required for monounsaturated fatty acid synthesis and which is induced to counteract the changes in membrane fluidity [14]. Mga1p has also been related to heat shock response and pseudohyphal growth [75]. The general stress response transcription factor Msn2p was also present in some conditions indicating cells are responding to stress [42].

2.3. Temperature responsive genes shared between cold and heat stresses

To assess the presence of general mechanisms between heat and cold in each of the assayed strains, we studied the intersection of DEG genes at low versus high temperature for each of the strains. Such a common stress mechanism may start general stress signaling cascades involved in damage repair to prevent cell death provoked by abrupt temperature changes [76]. Table 2 shows the number of common DEGs between low and high temperature with reciprocal or opposite expression behavior in the three strains. It can be observed a consistent positive correlation between gene expression under heat and cold stress conditions. In general, a higher number of the common genes between cold and heat conditions presented the same expression tendency in the three strains, suggesting that our hypothesis of a general mechanism between heat and cold could be valid.

ADY5 had a great number (49) of genes downregulated in both temperature conditions, mainly related with the transport of ammonium, acetate and organic acids, such as carboxylic acid (Table 2 and Table S5). It is striking the presence of the heat shock proteins *HSP26* and *HSP30* within the set of genes down-regulated in cold and up-regulated in heat. *HSP30* is a protein induced by heat shock and is a regulator of the H(+)-ATPase *PMA1* [77] while *HSP26* is a small heat shock protein (sHSP) with chaperone activity that suppress unfolded proteins aggregation [78]. Pma1p is important for maintenance of normal pH homeostasis in yeast [79]. Heat shock induces a substantial decrease (~1 pH unit) in cytoplasmic pH [69] leading to a perturbation on the redox regulation in the cytoplasm. Furthermore, recent studies have demonstrated that *HSP30* gene was also upregulated in cells subjected to high levels of oxidation [80]. Despite the *HSP30* up-regulation, we did not find its protein to be more abundant in yeast exposed to heat in our previous proteomic study [8]. It was postulated that stress might have a differential impact on the half-life time of *HSP30* mRNA and its protein level because the increase of ROS enhances Hsp30p degradation, maintaining high levels of its transcripts over protein molecules [80,81].

Table 2
Number of genes exhibiting reciprocal or inverse transcriptional responses comparing low and high temperature in each of the strains.

Number of genes	ADY5	CEN.PK113-7D	Ethanol Red
cold up and heat up	9	3	1
cold down and heat down	49	76	37
cold up and heat down	0	1	6
cold down and heat up	5	2	4

On the other hand, Hsp26p co-aggregates with misfolded proteins acting as first line of defense against proteotoxic stress, which is elevated during high temperatures exposures [66,78,82].

Regarding Ethanol Red, 37 genes were commonly down-regulated in both cold and heat (Table 2). Among them, categories related to anion transmembrane transport and arginine biosynthetic process (*ARG1*, *ARG3* and *CPA2*) were overrepresented (Table S5). These finding are in concordance with the proteomic analysis of this strain [8] and could potentially represent a mechanism to acquire thermotolerance which has been also described in other yeast species, such as *Kluyveromyces marxianus* grown at 45 °C [83]. The reduction of arginine production by downregulating its biosynthesis as well as the decrease in some precursor such as citrulline lead to glutamate conservation and proline production. Proline is the main metabolite of arginine metabolism and its accumulation is related with thermal stress protection by protein and membrane stabilization, lowering the DNA melting temperature and reactive oxygen species scavenging [84–86].

The lab strain CEN.PK113-7D presented a huge number (76) of shared down-regulated genes between cold and heat mainly belonging to the super pathway aerobic respiration and electron transport chain (p-value = 0.0059). This was not surprising because the cultures were done in anaerobic conditions. On the other hand, a novel cold adaptation mechanism by switching from respiratory to fermentative metabolism when cells are growing in a cold condition has been recently reported [87]. Authors propose that this switch may be an adaptation in order to save energy and delay intracellular freezing in cold environments. This mechanism has been described in the phylogenetically distant Antarctic yeast *Rhodotorula frigidialcoholis* but it can be an example of convergent

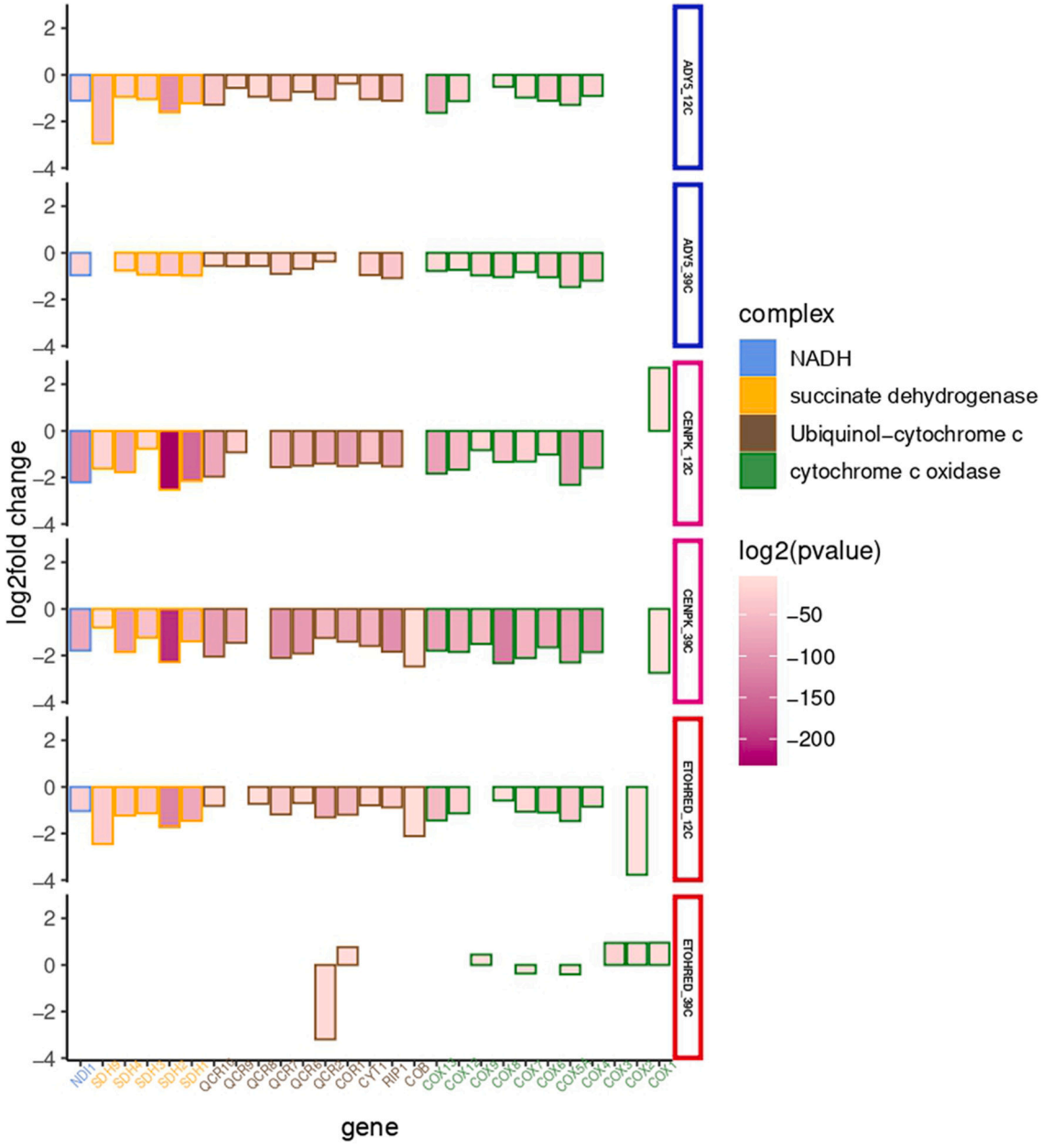

Fig. 5. Electron transport chain expression in ADY5, CEN.PK117-3D and Ethanol Red at 12 and 39 °C. The genes with a significant ($p < 0.05$) differential expression ($\geq \pm 1.5$ log2fc) are represented with bars. For the bars, the deeper the colour, the lowest the p-value. No bar indicates genes that are not differentially expressed in this condition. The genes belonging to the same protein complex are indicated in the same colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adaptation mechanism.

In order to know more in detail what was occurring in the electron transport chain in the three strains, we performed an analysis of the genes of this pathway (Fig. 5). As it can be seen, an important number of genes belonging to the four protein complexes were downregulated in the three strains in sub-optimal temperature conditions but mainly in cold condition. CEN.PK113-7D was the strain that presents the most pronounced downregulation of almost the complete set of these genes.

2.4. Transcriptomic and proteomic thermal response correlation

To determine differences at the proteomic level as a function of the temperature, SWATH analysis was also performed in the same samples used for the RNAseq analysis [8]. By using the available proteomic dataset, the log2 fold change for proteins was calculated (Table S6) and a Pearson correlation between the detected protein levels and their corresponding gene expression was performed for each strain and temperature (Fig. 6).

As a whole, all the conditions presented a positive correlation between both datasets ranging from 0.39 to 0.63. In addition, the correlation values (R) at low temperature were higher than those obtained at 39 °C for the three strains. It has been recently described in a high temperature growth analysis (37, 42 and 46 °C) the poor correlation between the transcriptome and the proteome under severe stress conditions [66]. In this study, the R values diminished as the temperature increased and authors propose protein aggregation, degradation, and impaired translation as possible processes that might explain the unmodified concentration of proteins despite the increased transcription of their respective mRNAs [66,88,89]. In addition, at higher temperatures the proteins are less stable so, in order to counteract this effect, transcriptomic upregulation is needed to keep stable the protein level and maintain the metabolic processes active [66].

Another likely explanation for the transcriptome and proteome differences is post-transcriptional regulation. The weak correlation at high temperature between the transcriptome and the proteome in some conditions could imply a major role for regulation at the post-transcription level. Similar results has been previously reported in other organisms [90,91].

Moreover, when the analysis was performed using the significant changes at both proteomic and transcriptomic level data (blue) the correlation values at both low and high temperature increased. In general, both industrial strains, ADY5 and Ethanol red, were the strains with the best correlation gene expression/protein abundance at both temperatures.

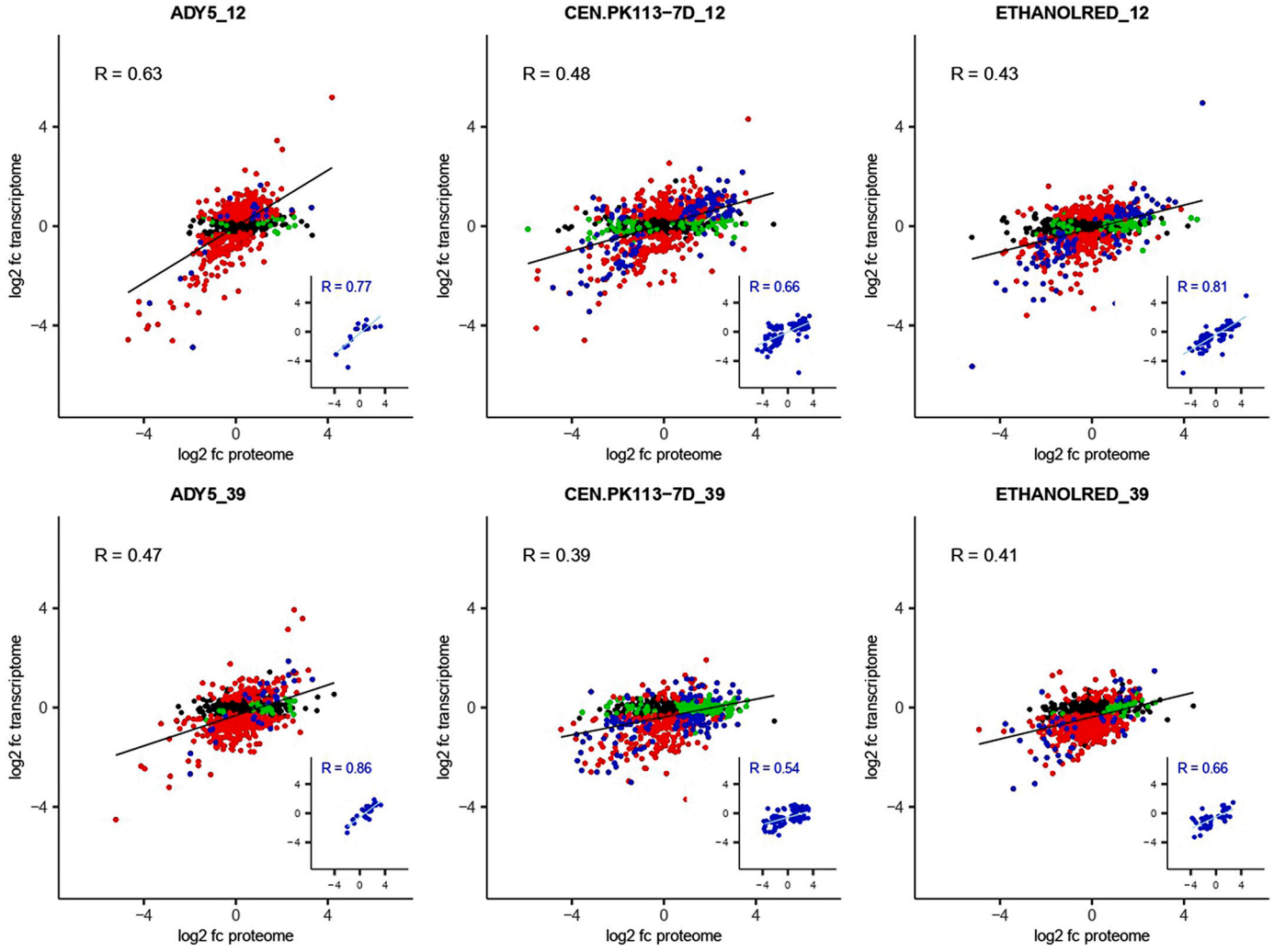

Fig. 6. Transcriptomic and proteomic temperature stress response correlations. Comparison of the transcriptome and proteome of cold and heat stressed yeast. We defined four different categories: differentially regulated proteins in green; differentially expressed genes in red; genes/proteins unchanged in black and significant changes at both proteomic and transcriptomic level in blue. (R, Pearson correlation). All *p*-values for the Pearson correlation were < 2.2e-16 (virtually 0), except for Ethanol Red at 39 °C (0.005). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Strain-specific temperature fingerprint

In order to analyze the variability of the samples in complex omic-scale, we used the DEcomposing heterogeneous Cohorts using Omic data profiling (DECO) tool [92]. The method identifies genes belonging to specific phenotypic conditions based on a recurrent differential analysis integrated with a non-symmetrical correspondence analysis. Thus, we have identified the characteristic set of genes in each of the strains at a given temperature, the so-called strain-specific temperature fingerprint (Fig. 7 and Table S7). These specific genes of each strain at each temperature do not have to be necessarily differentially expressed; it is what would actually separate a strain from the others in the selected conditions.

Fig. 7 represents a summary of the three strains in all the temperatures assayed showing the functional categories of each temperature and strain. As it can be seen, at low temperature, ADY5 is characterized by the metabolic activity of the pyruvate descarboxilase, mainly related with glycolysis; CEN.PK113-7D is mainly separated by carbohydrates transport while, in Ethanol Red, oxidoreductase activity is highlighted. Regarding heat, the three strains stand out for functional categories related with oxidoreductase activity, antioxidant activity and membrane transport. To get a more concrete fingerprint, one ought to step down to the gene level instead of the functional category. Finally, at optimum temperature, the activities were related with growth and proliferation but chaperone binding in ADY5 and proteasome-activating ATPase activity in CEN.PK113-7D can be highlighted. This dissecting method of strain dependent expression constitutes a novelty which could be applied in future studies with higher number of non-optimal temperature tolerant strains, and thus, determine which processes or genes define the expression of these strains under the given conditions. Likewise, this approach might hold an important value for identification or developing yeast with improved features for industry [93].

3. Conclusions

As a summary, by using our approach we show the huge complexity of the transcriptome remodeling related with growth at non-optimum temperatures, as well the paramount importance to analyze different strains to come to significant biological conclusions. Worth noting that low temperature exerted a stronger transcriptional response in the three strains which was concordant also with a greater proteome remodeling at low temperature previously described for the three studied strains [8].

We have also identified a possible convergent mechanism of adaptation, the metabolic switch from respiration to fermentation, recently described in the extremophilic yeast *Rhodotorula frigidialcoholis* [87]. This strategy is key mainly in cold environments but also seems to be

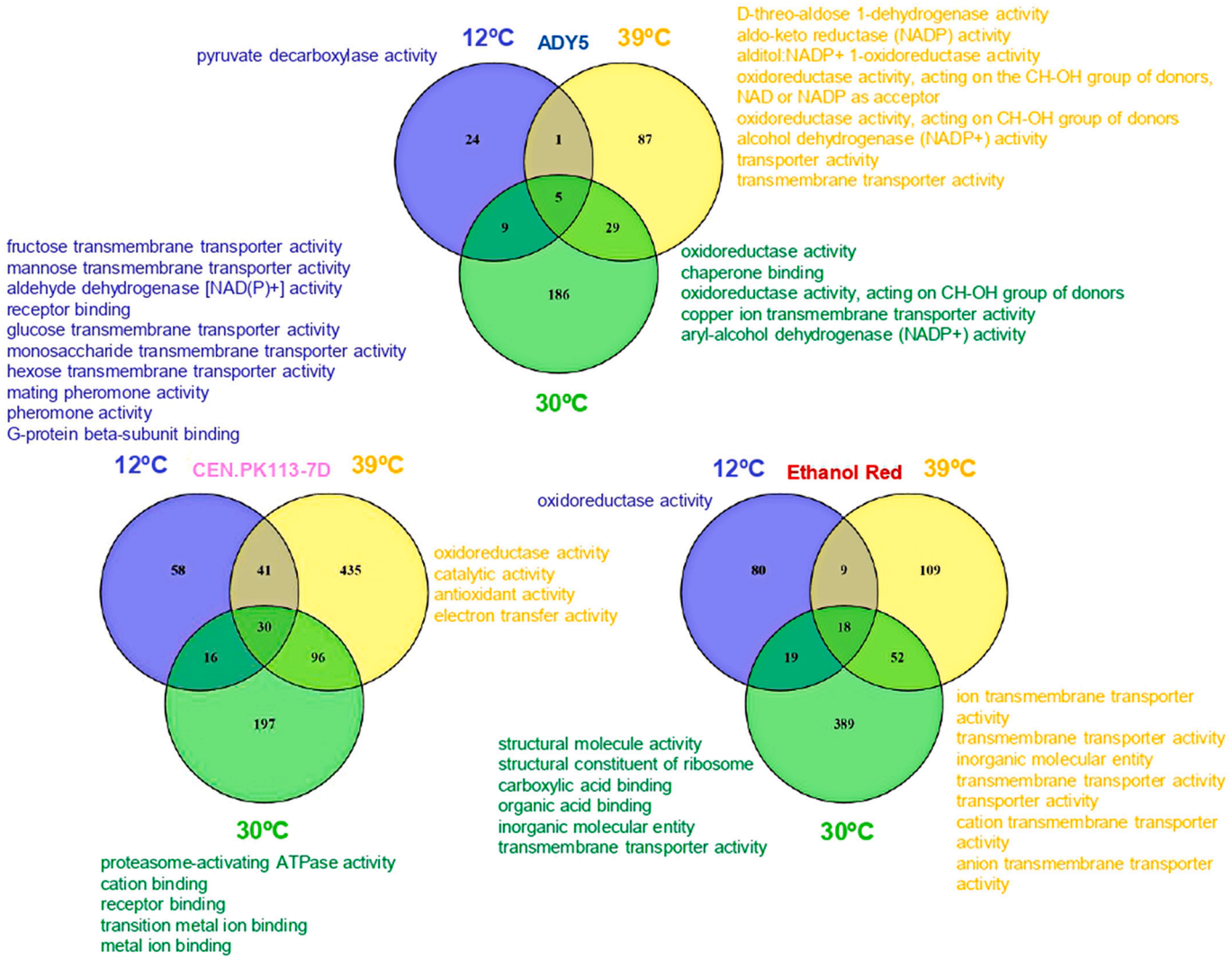

Fig. 7. Strain-specific temperature fingerprint obtained by DECO tool. Venn diagrams to show the overlap of the characteristics genes of the three strains at the three tested temperatures (12, 30 and 39 °C). The main significant ($p \leq 0.01$) functional categories of each condition are highlighted in different colors as a function of the temperature in each strain.

important in heat.

We have also stepped down to the level of key genes for adaptation to non-optimal temperatures. The clearer example is *OPI3*, which proved to be a gene with paramount importance in cold environments. However, we have also identified the genes *YOL014W* and *EFM6* to be involved in cold adaptation. Further studies are required in order to elucidate the role of both genes in this low temperature adaptation.

Furthermore, proteome changes under heat temperature shock do not correlate very well with gene regulation, which could be due to the aim to maintaining the levels of proteins by increasing the expression levels of the genes.

DECO tool has proven to be a complementary and interesting strategy to be applied within complex multiomic data in order to elucidate the main characteristics of a given strain in different conditions, such as thermal stress. This tool could be applied in future studies in order to identify new yeast with interesting capacities for the industry, based on similarity with already studied strains.

In conclusion, the wine/beer and bio-energy industries spend a huge amount (30–60%) of their total energy requirement to control the temperature of the process. In this sense, temperature is a key factor determining the operational costs of industrial fermentation processes. Therefore, better-adapted yeast strains able to grow in non-optimal temperatures provides an opportunity to improve the production process in economical and bio-sustainability terms.

4. Material and methods

4.1. Yeast strains and media

The yeast strains used in this work were *Saccharomyces cerevisiae* Cross Evolution™ (ADY5) (Lallemand Inc., Canada), a commercial wine strain, *S. cerevisiae* Ethanol Red® (Fermentis, S.I. Lesaffre, France), a commercial bioethanol strain, and the haploid laboratory strain *S. cerevisiae* CEN.PK113-7D (Fungal Biodiversity Centre, Utrecht, The Netherlands). Working stocks were prepared by cultivation in YPD medium (glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}). After addition of 30% (*v*/v) glycerol, culture aliquots were stored in sterilized Eppendorf tubes at −80 °C.

Inoculum for the chemostat cultivations were grown aerobically at 220 rpm at 30 °C in 2 L Erlenmeyer flasks containing 400 mL of filter sterilized medium containing per L: 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 15 g glucose.H_2O, 1 mL of trace element solution, and 1.0 mL vitamin solution. Trace element and vitamin solutions were prepared as described by Verduyn et al. [94]. The medium for anaerobic chemostat cultivation contained per L: 5.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.5 g·$MgSO_4{\cdot}7H_2O$, 22.0 g D-glucose·H_2O, 0.4 g Tween80, 10 mg ergosterol, 0.26 g antifoam C (Sigma-Aldrich, Missouri, USA), 1.0 mL trace element solution, and 1.0 mL vitamin solution. The medium was filter sterilized using a 0.2 μm Sartopore 2 filter unit (Sartorius Stedim, Goettingen, Germany).

4.2. Chemostat cultivations

All chemostat cultivations were carried out at a dilution rate of 0.030 ± 0.002 h^{-1} in 7 L bioreactors (Applikon, Delft, The Netherlands) equipped with a DCU3 control system and MFCS data acquisition and control software (Sartorius Stedim Biotech, Goettingen, Germany). The reactor vessels were equipped with norprene tubing, to minimize the diffusion of oxygen into the vessels, and were sterilized by autoclaving at 121 °C.

During chemostat operation the sterile feed medium was pumped into the reactor vessel at a constant flowrate using a peristaltic pump (Masterflex, Barrington, USA), such that the outflow rate of the culture broth was 120 ± 1 g·h^{-1}. The broth mass in the reactor was kept at 4.00 ± 0.05 kg, by discontinuous removal of culture into a sterile effluent vessel, via a pneumatically operated valve in the bottom of the reactor and a peristaltic pump, which were operated by weight control. Therefore, the complete reactor was placed on a load cell (Mettler Toledo, Tiel, The Netherlands). In addition, the effluent vessel was placed on a load cell of which the signal was continuously logged for accurate determination of the dilution rate of the chemostat and manual adjustment of the medium feed rate if needed.

The cultivations were carried out at temperatures of either 12.0 ± 0.1 °C, 30.0 ± 0.1 °C or 39.0 ± 0.1 °C, by pumping cooled or heated water through the stainless-steel jacket surrounding the bottom part of the reactor vessel, using a cryothermostat (Lauda RE630, Lauda-Königshofen, Germany). The water temperature of the cryothermostat was controlled by using the signal of a Pt 100 temperature sensor inside the reactor, for accurate measurement and control of the cultivation temperature. Anaerobic conditions were maintained by continuously gassing of the reactor with nitrogen gas at a flowrate of 1 SLM (standard liter per minute) using a mass flow controller (Brooks, Hatfield, USA). Also, the feed medium was kept anaerobically by sparging with nitrogen gas. The nitrogen gas was sterilized by passing through hydrophobic plate filters with a pore size of 0.2 μm (Millex, Millipore, Billerica, USA). The culture broth in the reactor was mixed using one 6-bladed Rushton turbine (diameter 80 mm) operated at a rotation speed of 450 rpm. The pH was controlled at 5.00 ± 0.05 by automatic titration with 4 M KOH. The bioreactor was inoculated with 400 mL of pre-culture and subsequently operated in batch-mode, allowing the cells to grow at the same temperature as the chemostat culture and to achieve enough biomass at the start of the chemostat phase. The exhaust gas from the chemostat was passed through a condenser kept at 4 °C and then through a Perma Pure Dryer (Inacom Instruments, Overberg, The Netherlands) to remove all water vapor and subsequently entered a Rosemount NGA 2000 gas analyzer (Minnesota, USA) for measurement of the CO_2 concentration. When the CO_2 level of the exhaust gas during the batch cultivation dropped significantly, close to the level after the pre-culture inoculation, this indicated the end of the batch phase. Thereafter the culture was switched to chemostat mode. Sampling was carried out during steady state conditions, after stable values of the CO_2 level in the exhaust gas and the biomass dry weight concentration were obtained. Triplicate samples were taken from each chemostat cultivation approximately every 48 h during the steady state for measurement of cell dry weight and transcriptomic analysis. The steady-state of the chemostat cultures was confirmed by the stable CO_2 off gas profile and steady dry-weight measurement.

For RNA analysis 10 g in triplicate of chemostat broth was immediately cooled down to around 4 °C after sampling by pouring into a tube containing cold steel beads. After cold centrifugation, the cell pellets were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

4.3. Genomic DNA extraction and whole genome sequencing

Genomic DNA was extracted from ADY5 and CEN.PK113-7D strains [95]. Sequencing libraries were prepared using the TruSeq DNA PCR-free kit. Sequencing was performed using the Illumina Miseq system; with paired-end 250 bp. ADY5 and CEN.PK113-7D sequences are available at NCBI repository under the BioProject ID PRJNA830720. Ethanol red sequence was available at SRA NCBI sample SRR2002842. Adaptors were removed from reads with Trimmomatic [96] and reads were trimmed with Sickle v1.2 [97] with a minimum quality value per base of 28 at both ends and a minimum read length of 180 bp.

4.4. Genome mapping and variant calling

Sequencing reads were mapped against the reference *S. cerevisiae* S288C genome (version R64-2-1) using bowtie2 v2.3.0 [98] with default parameters. The read depth (RD) or coverage "per base" was then obtained using bedtools v2.17.0 (Quinlan and Hall, 2010). The obtained coverage files were processed by a sliding windows strategy with a windows size of 1 kb moving by 1 kb. This permitted an average

coverage value to be obtained every 1000 positions. Copy number variations (CNV) were called by CNVnator [99]. For SNVs and small indels assessment, the breseq pipeline was used [100] with option -p –polymorphism-frequency-cutoff of 0.45. In particular, we applied the command SUBSTRACT from gdtools to obtain and annotate those mutations that are exclusive of each of the strains with respect to the others.

4.5. RNA sequencing

RNA isolation was performed with the High Pure RNA Isolation kit (Roche Applied Science, Germany). After oligo (dT) mRNA purification, RNAseq libraries were generated with the TruSeq Stranded mRNA Library Preparation Kit (Illumina, CA, USA). A pool of the libraries was sequenced on a NextSeq Sequencing System from Illumina (2 × 75 bp). Raw reads data files were deposited on the European Nucleotide Archive under the project's accession number PRJEB47443.

4.6. RNAseq analysis and pipeline

Sequence reads were mapped *to S. cerevisiae* pangenome published by [101] genome using Bowtie2 (Bowtie2 v. 2.2.9 –local) [98]. After that, we compressed and sorted the alignments using SAMtools v-1.4.1 (Li H., et al. 2009. The Sequence alignment/map (SAM) format and SAMtools. Bioinformatics, 25, 2078-9). Read counts for each gene were obtained using HTSeq-Count (HTSeq-0.6.1p1, −m union –a 10) [102]. Python framework to work with high-throughput sequencing data). We obtained on average 2.7 million reads per sample, with a range of 1.4 to 4.5 million reads. We observed a mean of 284 reads per gene across all 27 samples.

PCA of the samples according to the gene expression was built using DESeq2 function dds, which normalizes and adjusts the sample counts to a negative binomial distribution, followed by a variance stabilizing transformation function also included in this R package. From that normalized data, function contrast was employed to obtain the differentially expressed genes between pairs of conditions. Differentially expressed genes (DEG) were used in Gene Ontology (GO) enrichment analysis as implemented in *Saccharomyces* Genome Database [103] (p value $\leq$0.01). A Bonferroni correction was applied to correct for multiple comparisons.

Heatmap was obtained by hierarchical clustering by the Ward method of the GO terms p-values euclidean distance matrix, using the R function *heatmap.2* included in the package *gplots*. Previously, all p-values higher than 0.01 (not significant) were made equal to 0.01 for clarity in the graphical representation.

The main features differentiating the samples were identified by means of the DECO R package [92]. This pipeline includes a previous normalization by voom method from limma R package. After that, we performed a supervised recurrent-sampling differential analysis (also from limma) for each of the temperatures assayed, dividing the samples according to the different strains. Fisher's combined probability test is applied to each final feature vector of p-values to obtain a Standard Chi Square to summarize the positive differential signal for each feature. Finally, on differential events from significant features, DECO performs a non-symmetrical correspondence analysis, to improve the final sample stratification. This allows DECO to identify those genes better differentiating one strain from the others.

We used the proteomic dataset available from a previous work [8] in order to perform analysis of correlation with RNAseq data. Statistical significance of proteomics data was assigned by using a two-sided t-test from the normalized area of the identified peaks. P values below 0.05 were considered significant. Fold change was used for the comparison with the transcriptomic data. Pearson correlations of every relevant fold change were calculated. Proteomic data is available via ProteomeXchange (PXD016567).

4.7. Construction of mutant for gene validation

The two copies of the genes were deleted using the short flanking homology (SFH) method [104]. The primers used for amplification of the KanMX4 and HphMX4 cassette from the plasmids pUG6 and pAG32, respectively, presented 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragments were used to transform the three studied strains using the lithium acetate procedure [105]. Transformants were selected by resistance to G418 and hygromycin, and correct deletion cassette integration was confirmed by diagnostic PCR using the primers upstream and downstream of the deleted region (Table S8). Growth was monitored by determining optical density at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) as previously described [106].

4.8. Statistical analyses

The results are expressed as mean and standard deviation of three independent biological replicates. To evaluate statistical significance in the growth experiments, tailed t-student tests were applied. The asterisk (*) indicates statistically significant differences with p-value $\leq$0.05. Phenotypic data were fitted to the reparametrized Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0.

Credit author statement

EGR, JAR, KYFL and TP have performed all the experiments in the lab. EGR, JAR performed the genomic analysis of the different strains. KYFL and WG were in charge of the industrial fermentations. TP, JT and LD mainly conducted the proteomic experiment. EGR, AQ and JMG have designed, discussed the experimental work and have also written and revised the manuscript.

Declaration of Competing Interest

The author declares that there are no competing interests regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110386.

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