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REVIEW

Omics analysis of acetic acid tolerance in *Saccharomyces cerevisiae*

Peng Geng[1,2] · Liang Zhang[1,2] · Gui Yang Shi[1,2]

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Abstract Acetic acid is an inhibitor in industrial processes such as wine making and bioethanol production from cellulosic hydrolysate. It causes energy depletion, inhibition of metabolic enzyme activity, growth arrest and ethanol productivity losses in *Saccharomyces cerevisiae*. Therefore, understanding the mechanisms of the yeast responses to acetic acid stress is essential for improving acetic acid tolerance and ethanol production. Although 329 genes associated with acetic acid tolerance have been identified in the *Saccharomyces* genome and included in the database (http://www.yeastgenome.org/observable/resistance_to_acetic_acid/overview), the cellular mechanistic responses to acetic acid remain unclear in this organism. Post-genomic approaches such as transcriptomics, proteomics, metabolomics and chemogenomics are being applied to yeast and are providing insight into the mechanisms and interactions of genes, proteins and other components that together determine complex quantitative phenotypic traits such as acetic acid tolerance. This review focuses on these omics approaches in the response to acetic acid in *S. cerevisiae*. Additionally, several novel strains with improved acetic acid tolerance have been engineered by modifying key genes, and the application of these strains and recently acquired knowledge to industrial processes is also discussed.

✉ Liang Zhang
zhangl@jiangnan.edu.cn

[1] The Key Laboratory of Industrial Biotechnology of Ministry of Education, Jiangnan University, Wuxi, China

[2] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi 214122, China

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Introduction

Lignocellulosic materials are rapidly becoming a major source of bioethanol because they are abundant, renewable, and uncompetitive with food resources. However, their pre-treatment usually generates many inhibitory compounds that hamper microorganism growth, such as formic acid, acetic acid and furfural (Fernandes et al. 2005; Luo et al. 2002). Since lignin and hemicellulose are highly acetylated, acetic acid is produced during the pre-treatment process, and it remains in the resultant hydrolysate (Chesson et al. 1983). Acetic acid also causes intracellular acidification, leading to energy depletion (Pampulha and Loureiro-Dias 2000) and inhibition of cell growth and metabolism during lignocellulosic fermentation. This weak acid is one of the main inhibitors in lignocellulosic hydrolysates.

Understanding the physiological mechanisms of *Saccharomyces cerevisiae* is a basic requirement for enhancing acetic acid tolerance during fermentation and improving product quality, such as balsamic vinegar and wine (Solieri and Giudici 2008; Vilela-Moura et al. 2011). Acetic acid tolerance is controlled by multiple genes, and even in a relatively simple model organism such as *S. cerevisiae*, precisely determining the genes involved and their interactions is highly challenging. Part of the reason is the difficulty in narrowing phenotypes to single genes and in detecting and quantifying epistasis, the contribution of variable quantitative traits and linked quantitative trait loci (Wilkening et al. 2014). Thus, the cellular stress responses to acetic acid

remain unclear, which makes it difficult to improve this organism by genetic engineering.

After decades of post-genomic research, several approaches have been developed in *S. cerevisiae* at a genome-wide scale, including DNA microarray analysis, functional screening of non-essential gene deletion collections, whole genome sequence analysis, and inverse engineering. By using these technologies, we could dissect acetic acid tolerance and other complex traits in yeast. This review gives an overview of high-throughput technologies used to decipher acetic acid tolerance in *S. cerevisiae*. We discuss the advantages and challenges of these approaches, the novel genes identified, and novel strains constructed by modifying some key genes.

General mechanisms of acetic acid tolerance

In low pH conditions (<4.76), acetic acid is in an undissociated (protonated) state that can enter the cell through the Fps1p channel or by simple diffusion (Mollapour and Piper 2007). Once in the cytoplasm, acetic acid dissociates into acetate anions and protons, leading to cytoplasmic acidification and inhibition of some important metabolic processes (Arneborg et al. 2000). *S. cerevisiae* is sensitive to acetic acid in the presence of glucose, which restrains acetic acid depletion. Yeast can undergo programmed cell death triggered by internal and external stimuli including acetic acid (Giannattasio et al. 2013; Ludovico et al. 2001).

Nevertheless, yeast can survive in the presence of low concentrations of acetic acid. Acetate anions can be converted to acetyl-CoA by peroxisomal or cytosolic acetyl-CoA synthetases. Then acetyl-CoA enters the tricarboxylic cycle or glyoxylate cycle (Lee et al. 2011; Rolland et al. 2002; Vilela-Moura et al. 2008). Yeast adapted to acetic acid display many physiological alterations, including cell wall and membrane reorganization, pH recovery, efflux of anions, and detoxification (Mira et al. 2010c). Two important enzymes are required to maintain the pH in the cytoplasm: the plasma membrane proton-pumping ATPase (PM-H^+-ATPase) and the vacuolar proton-pumping ATPase (V-H^+-ATPase). PM-H^+-ATPase is activated by weak acid, and pumps out protons in response (Carmelo et al. 1997). V-H^+-ATPase also contributes to the recovery and maintenance of cytosolic pH, and it is crucial for the other physiological processes (Kane 2006; Martinez-Munoz and Kane 2008). Meanwhile, the ATP-binding cassette (ABC) transporter Pdr12 can export some organic anions out of the cell (Holyoak et al. 1999) (Fig. 1).

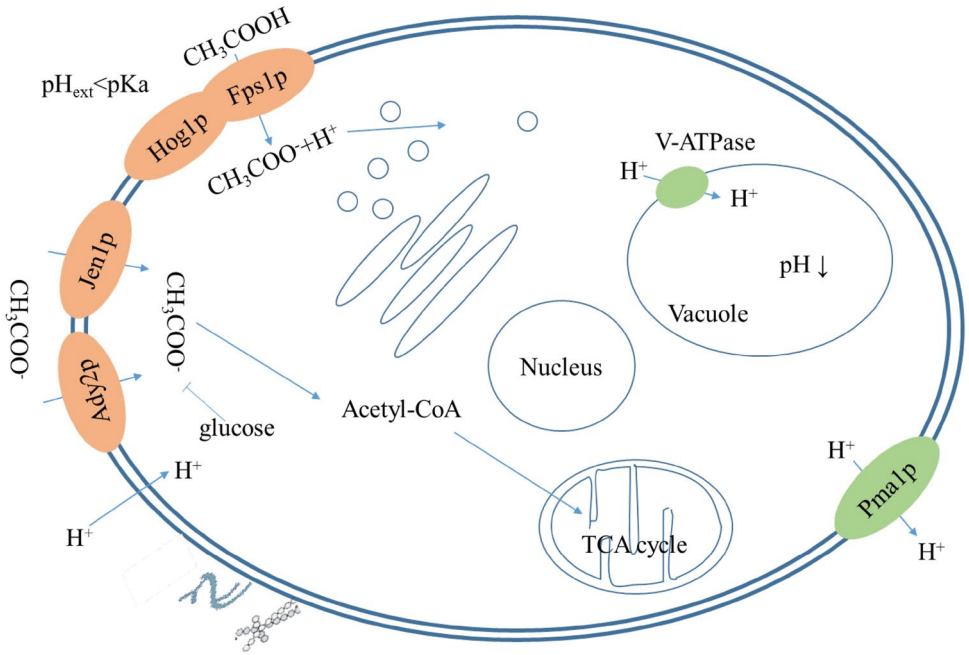

Fig. 1 Mechanisms of acetic acid stress in *S. cerevisiae*. When yeast cells are in acetic acid environment, the cell wall structure will reconstruct and plasma membrane lipid composition will reconfigure. Yeast can utilize acetate anion through Jen1p or Ady2p when glucose doesn't exist. At low pH, acetic acid enters cells in undissociated form by simple diffusion or through Fps1p. In the neutral cytosolic pH, acetic acid dissociated acetate anions and protons. Proton can be excluded out of the cytoplasm through Pma1p or V-ATPase to recover intracellular pH

Omics analysis of acetic acid tolerance

It is well known that most tolerance traits are controlled by multiple genetic loci, and omics analysis offers a promising approach for identifying the genetic basis of quantitative traits. Omics analysis can probe cells at a genome-wide scale to provide information on genome, transcriptome, proteome, and metabolome, due to the availability of complete genome sequences. Nowadays, more and more omics technologies are being used in the study of acetic acid tolerance in *S. cerevisiae*, and these are summarized below.

Functional genomics screening

With the development of high-throughput culturing technologies, many strains can be analysed to identify candidate genes involved in acetic acid tolerance. Gene deletion is the most common method deployed over the past 10 years. Kawahata et al. (2006) screened 4908 strains and identified genes involved in cell wall components that are important for responding to organic acids and low pH. Another group identified approximately 490 of the 650 determinants of tolerance to acetic acid by screening a ~5100 individuals from a haploid mutant collection for susceptibility phenotypes to acetic acid in non-essential deletion strains (Mira et al. 2010b). Candidate genes identified in these studies could be tested in genetic engineering experiments to obtain more robust strains.

Moreover, rapid molecular genetic manipulation technologies make it possible to screen strains overexpressing specific genes aimed at improving acetic acid tolerance. For example, Ma et al. (2015) screened strains transformed with an artificial zinc finger protein transcription factor (ZFP-TF) library and obtained strain ATCC4126 (Sc4126-MO1) that displayed improved acetic acid tolerance. They conducted further analysis and found three genes, *YFL040W, QDR3*, and *IKS1*, that are involved in the enhanced acetic acid tolerance, demonstrating the power of a synthetic ZFP-TF library for improving acetic acid tolerance. The employment of an artificial transcription factor can also facilitate the exploration of novel functional genes involved in stress tolerance. Functional genomes screening is performed by single gene deleted strains, but the essential genes are difficult to knock out. So, one drawback of this technique is the inconvenience of having to detect essential genes.

Genome analysis

In recent years, whole genome sequencing has substituted automated sanger sequencing for large-scale sequence data collection, mainly due to parallelization of the sequencing process. The available whole genome microarrays provide an enormous volume of inexpensive and accurate genome sequence data (Metzker 2010). By whole genome sequencing, four genes (*ASG1, ADH3, SKS1* and *GIS4*) associated with acetic acid tolerance were identified (González-Ramos et al. 2016). Also, whole genome sequence analysis of pooled segregants can be widely used to identify genetic determinants throughout the genome (Ehrenreich et al. 2010), such as those involved in sporulation efficiency (Ben-Ari et al. 2006), ethanol tolerance (Swinnen et al. 2012a), maximal ethanol accumulation capacity (Pais et al. 2013), low glycerol production (Hubmann et al. 2013a, b), high thermotolerance (Yang et al. 2013), survival at low pH (Fletcher et al. 2015), and high acetic acid tolerance (Meijnen et al. 2016). In these studies, one or more segregants were sequenced at a whole genome level to dissect the relationships between phenotypes and genotypes. Additionally, this technology provides a new way of scoring large numbers of genetic markers distributed throughout the genome, such as single nucleotide polymorphisms (SNPs) (Otero et al. 2010) and simple sequence repeats (SSRs) (Geng et al. 2016). By combining bioinformatics with whole genome high-throughput sequencing, we have personally identified genes with specific SNPs that are associated with acetic acid tolerance (unpublished data).

Approaches for whole genome sequencing allow using relatively low numbers of selected segregants to identify major genetic loci. However, validation of this methodology through the identification of all causative genes within a genome remains a challenge. Furthermore, application to a large number of individual segregants remains time-consuming and costly (Swinnen et al. 2012b).

Transcriptome analysis

Genome-wide changes in transcription occur in yeast cells during the early stages of the response to acetic acid stress that are believed to be important as a first step in adapting to an acidic environment. Through genome-wide transcriptome analysis, a comprehensive view of acetic acid tolerance can be achieved. This technology could be further deployed to search for transcription regulon factors. Indeed, several studies have reported transcriptional changes in *S. cerevisiae* cells exposed to acetic acid. These include the following various types of such changes: (a) two short time (Li and Yuan 2010; Mira et al. 2010a); (b) two long time (Abbott et al. 2007; Bajwa et al. 2013); (c) one between short and long time (Lee et al. 2015); and (d) one both short and long time (Kawahata et al. 2006). By studying genomic expression using DNA microarray analysis, Haa1p was found to regulate, directly or indirectly, the transcription of approximately 80% of acetic acid-activated genes. This suggests that Haa1p is the main player controlling the yeast response to acetic acid (Mira et al. 2010a).

Transcriptome analysis can also identify genes that are up- or down-regulated in the presence of acetic acid. Ismail et al. (2014) compared the transcriptomes of cells supplemented with metal ion cultures and untreated controls, and found that up-regulation of *FIT2, HXT1* and *TKL1* could enhance xylose consumption in the presence of acetic acid. An et al. (2015) screened a library of mutants overexpressing alleles of the TATA-binding gene *SPT15*, and identified two *S. cerevisiae* strains with enhanced tolerance to acetic acid with 58 up-regulated genes and 106 down-regulated genes.

In recent years, RNA sequencing has emerged as a novel high-throughput approach for transcriptomic profiling based on deep-sequencing technologies (Braconi et al. 2016; Nagalakshmi et al. 2008). RNA sequencing approaches are particularly suitable for comparative analysis aimed at evaluating structural and gene expression variability among different strains and species (Sardu et al. 2014). By RNA sequencing, Chen et al. (2016a) identified 184 consensus genes between strain YC1 and S-C1 in response to the distinct inhibitor resistance and found key transcription factors that regulate these consensus genes. And as a generally applicable method, RNA interference (RNAi) can be used for genome-scale engineering. Si et al. (2015) showed that three rounds of iterative RNAi screening led to the identification of three gene knockdown targets that acted synergistically to confer an engineered yeast strain with substantially improved acetic acid tolerance. In the near future, RNA sequencing is likely to provide an invaluable contribution to environmental toxicological studies.

Proteomic analysis

Among the various methods, proteomic analysis provides a powerful way to analyse the cellular protein profile in the presence of acetic acid. Ghaemmaghami et al. (2003) created a fusion library of open reading frames tagged with a high-affinity epitope, making it possible to analyse the expression of misannotated genes at a global level. In acetic acid-induced programmed cell death progress, Longo et al. (2015) analysed proteomic data from wild type and Δ*yca1* cells and found that carbohydrate catabolism, lipid metabolism, proteolysis and stress responses were the main functional roles. Lv et al. (2014) analysed the effect of toxic compounds including acids, furans, and phenols on yeast. In proteomic analysis, 194 and 215 unique proteins were identified as differently expressed proteins at lag phase and exponential phase, respectively.

Metabolomic analysis

Metabolomics has emerged as an important tool in many disciplines (Cevallos-Cevallos et al. 2009). Generally, metabolite concentrations are the result of complicated cellular mechanisms, including both transcriptional and translational regulation. Therefore, metabolome data reflects the metabolic state of the cell better than transcriptomic or proteomic data. It is therefore superior for understanding what has happened and is happening inside a cell during a given biological process (Weckwerth 2003). Metabolomic analysis has been used to identify the key metabolites and metabolic reactions involved in stress and to improve stress tolerance (Ding et al. 2012; Hasunuma et al. 2011; Nugroho et al. 2015). An et al. (2015) used metabolome profile analysis to reveal that the intracellular concentrations of five metabolites were increased and 102 were decreased in *S. cerevisiae* MRRC3252. They also found that deletion of the urea degradation gene *DUR1/2* and low levels of amino acids enhanced tolerance to acetic acid (An et al. 2015). Nugroho et al. (2015) conducted a metabolomics approach to investigate the effect of lactic acid-induced stress on metabolite pools in *S. cerevisiae*. And their results suggested that the addition of proline improves the specific growth rate and protects cells from acid stress by combating acid-induced oxidative stress.

Genetic manipulation for improving acetic acid tolerance

Omics approaches could provide the theoretical basis and definite genes for genetic modification in yeast. By functional genomics screening and genome wide analysis, many genes associated with acetic acid tolerance are involved in carbohydrate metabolism, protein folding, lipid metabolism, cell wall function and transport (Mira et al. 2010b). There are several examples of genetic manipulation to alter the cell structure and thereby improve acetic acid tolerance.

The plasma membrane is an important barrier in acetic acid tolerance. When acetic acid is present, the expression of *YRO2* is induced in the plasma membrane, and the Yro2 protein is believed to alleviate acetic acid-induced damage (Takabatake et al. 2015). Acetic acid can also enter the cell through the Fps1p channel. While deletion of fps1 can improve ethanol production and decrease acetic acid yield. Part of the reason was that the *fps1*Δ mutant might solve the occurring redox balance problems by reducing acetic acid (Zhang et al. 2007).

In the cytoplasm, acetic acid dissociates into acetate anions and protons, and the consumption of acetate and/or protection of intracellular proteins can improve acetic acid tolerance. Through genomic library screening, Chen et al. (2016b) found that the endogenous expression of Whi2 could be activated by acetic acid. So, overexpression of *WHI2*, encoding a 55 kDa cytoplasmic globular scaffold protein, can improve acetic acid tolerance and provide a protective effect in *S. cerevisiae*. And overexpression of

Table 1 The efforts to improve acetic acid tolerance in yeast

Strains	pH	Genes and manuscripts	Relation to omics	Effects of acetic acid compare to wild strains	Tolerance levels	References
S288c	4.8	*PEP3* ↑	Overexpression library screening	Shorter lag phase but does not alter growth rate	80 mM	Ding et al. (2015a)
S288c	4.8	*ACS2* ↑	–	Increased growth rate and shorter lag phase	140 mM	Ding et al. (2015b)
SR8	4.0	*ACE2* ↑	Transcriptome analysis	Improved tolerance to acetic acid and furfural	33 mM	Chen et al. (2016a)
SR8	4.0	*WHI2* ↑	Genomic library screening	Improved glucose and/or xylose fermentation under acetic acid stress	50 mM	Chen et al. (2016b)
BY4741	5.5	*JJJ1* ↓	–	Increased ethanol production and shorter lag phase	75 mM	Wu et al. (2016)
BY4741	1.2	*COX20* ↑	–	Improved tolerance to acetic acid and hydrogen peroxide-induced oxidative stress	75 mM	Kumar et al. (2015)
BY4741	3.5	*RTT109* ↓	–	Increased acetic acid tolerance and ethanol production rate	92 mM	Cheng et al. (2016)
D452-2	3.2	*OAZ1* and *TPO1* double disruption and *SPE3* ↑	RNA-sequence analysis	Increased spermidine content and shorter lag phase	67 mM	Kim et al. (2015)
CEN.PK113-13D	4.5	*HAA1* ↑ and the $Haa1^{S135F}$ mutant strain	SNP analysis	Mutant led to an increased recruitment to Haa1 target genes	50 mM	Swinnen et al. (2017)

↑: Indicating that the gene is overexpressed

↓: Indicating that the gene is deleted

ACS2, an acetyl-coenzyme A synthetase, also improves resistance to acetic acid during fermentation (Ding et al. 2015b). Wu et al. (2016) found that higher long chain fatty acid concentrations, intracellular trehalose levels, and CAT activity are important factors contributing to the improved acetic acid tolerance of *jjj1*Δ mutants.

Mitochondria are the epicentre of energy production in the cell, and an important organelle for tolerance to acetic acid. Kumar et al. (2015) overexpressed *COX20*, a mitochondrial cytochrome c oxidase chaperone, which improved tolerance to acetic acid during fermentation.

The biogenesis and fragmentation of the vacuole is dependent on vesicle–vesicle and vesicle–vacuole fusion and fission events. By screening overexpression library, overexpression of *PEP3, VAM6*, or *VPS3* increases the number of fragmented vacuoles, and overexpression of *PEP3* in particular shortens the lag phase associated with stress tolerance without altering the growth rate (Ding et al. 2015a).

The nucleus also plays a role in acetic acid tolerance. Chromatin dynamics controlled by various histone modification enzymes is important for stress-responsive genes, and histone H3/H4 acetylation is an important histone modification. By screening 345 single point mutations in H3 and H4 histone libraries, Liu et al. (2014) identified two mutants, H3K37A and H4K16Q, that showed improved ethanol fermentation ability under acetic acid stress conditions. And they further analysed acetic acid tolerance and ethanol fermentation capacity of the mutants by genome-wide transcriptional profiling. As we know, *RTT109* is a histone acetyltransferase for the acetylation of histone H3. Cheng et al. (2016) fought that the absence of *RTT109* not only activates transcription of stress responsive genes, but also improves resistance to oxidative stress, which ultimately contributes to improved acetic acid tolerance in *S. cerevisiae*.

To date, many studies have focused on laboratory strains, but few have investigated industrial strains or naturally occurring organisms isolated from the environment. This may be because industrial strains have a more complex genome, since they are often diploid, polyploid, or aneuploid. In general, a true and stable haploid strain is difficult to obtain. Furthermore, phenotypic screening can be laborious and time-consuming, hence it is difficult

to directly identify some industrially relevant traits using bulk segregant approaches (Swinnen et al. 2012b). However, several successful strategies using high-throughput technologies can now be applied to industrial strains. In one study, highly ethanol tolerant industrial strains were investigated and three causative genes (*MKT1, SKS2* and *APJ1*) identified by pooled-segregant whole genome sequence analysis (Swinnen et al. 2012a). Another study crossed haploid segregants of a strain with an unusually high acetic acid tolerance and a reference industrial strain, resulting in the identification of three new causative alleles (*GLO1, DOT5, CUP2*) that determine high acetic acid tolerance (Meijnen et al. 2016). Such methods are therefore capable of dissecting specific phenotypes at a whole genome scale, and the knowledge obtained can be used to improve acetic acid tolerance in industrial strains (Table 1).

Perspectives

Several molecular factors have been identified that are related to acetic acid and modified to engineer improved strains. These include strains overexpressing *HAA1* and *GRX5* (Fang et al. 2015; Sakihama et al. 2015; Swinnen et al. 2017), strains in which *FPS1* and *PHO13* are deleted (Sakihama et al. 2015), and 52 individual deletion strains identified by screening a deletion mutant library. However, because of the complex mechanisms involved in the response to acetic acid in yeast, a global molecular analysis of strains by whole genome sequencing and/or omics technologies is required to assess weak acid tolerance. Fortunately, recent advances in high-throughput techniques, including genomics, transcriptomics, proteomics, and metabolomics, are providing us with a vast amount of biological data to help us unravel these biological processes. Furthermore, these results can be used to engineer more robust strains with improved stress resistance.

Whole genome sequencing can certainly initiate a better understanding of stress tolerance, but it cannot distinguish between trait-relevant and trait-irrelevant molecular differences. Bansal (2005) summarized the limitations in genomics and proteomics approaches as follows: a lack of available gene-functionality information in wet-lab data, a lack of computer algorithms to explore the vast amount of data with unknown functionality, a limited knowledge of protein–protein and protein–DNA interactions, and a lack of knowledge concerning the temporal and transient behaviour of genes and pathways. Interestingly, genome-wide profiles are rarely in agreement, apparently due to different experimental conditions, including the strains used, the pH of the medium, and the acetic acid concentration. However, all data are valuable for both reinforcing previous findings and understanding regulatory networks (Mira et al. 2010c). Further elucidation of the molecular mechanisms of acetic acid tolerance will likely require a combination of high-throughput approaches and other methods, such as gene manipulation techniques including CRISPER/Cas9, systems metabolic analysis and evolution engineering.

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