

Molecular fingerprints of conazoles via functional genomic profiling of *Saccharomyces cerevisiae*

Miao Guan^{a,b,*}, Pu Xia^a, Mingming Tian^a, Dong Chen^{a,c}, Xiaowei Zhang^{a,*}

^a State Key Laboratory of Pollution Control & Resource Reuse, School of the Environment, Nanjing University, 163 Xianlin Ave., Nanjing, Jiangsu 210023, China

^b Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Rd., Nanjing, Jiangsu 210023, China

^c Jiangsu Provincial Academy of Environmental Science, 176 North Jiangdong Rd., Nanjing, Jiangsu 210036, China

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ABSTRACT

Conazoles were designed to inhibit ergosterol biosynthesis. Conazoles have been widely used as agricultural fungicides and are frequently detected in the environment. Although conazoles have been reported to have adverse effects, such as potential carcinogenic effects, the underlying molecular mechanisms of toxicity remain unclear. Here, the molecular fingerprints of five conazoles (propiconazole (Pro), penconazole (Pen), tebuconazole (Teb), flusilazole (Flu) and epoxiconazole (Epo)) were assessed in *Saccharomyces cerevisiae* (yeast) via functional genome-wide knockout mutant profiling. A total of 169 (4.49%), 176 (4.67%), 198 (5.26%), 218 (5.79%) and 173 (4.59%) responsive genes were identified at three concentrations (IC_{50} , IC_{20} and IC_{10}) of Pro, Pen, Teb, Flu and Epo, respectively. The five conazoles tended to have similar gene mutant fingerprints and toxicity mechanisms. "Ribosome" (sce03010) and "cytoplasmic translation" (GO: 0002181) were the common KEGG pathway and GO biological process term by gene set enrichment analysis of the responsive genes, which suggested that conazoles influenced protein synthesis. Conazoles also affected fatty acids synthesis because "biosynthesis of unsaturated fatty acids" pathway was among the top-ranked KEGG pathways. Moreover, two genes, YGR037C (acyl-CoA-binding protein) and YCR034W (fatty acid elongase), were key fingerprints of conazoles because they played vital roles in conazole-induced toxicity. Overall, the fingerprints derived from the yeast functional genomic screening provide an alternative approach to elucidate the molecular mechanisms of environmental pollutant conazoles.

1. Introduction

Conazole fungicides, which are among the top ten classes of pesticides currently used and are the second most consumed fungicides in the global market (Consulting, 2016; Fenner et al., 2013). Conazole fungicides are widely used in agriculture (field crops, fruit trees and vegetables) for the control of fungi (Fletcher, 2000). Conazoles have frequently been detected in the environment at levels of nanogram to microgram per liter (ranged from 0.004 to 81) in surface waters from different continents and was increasing by years (Bradley et al., 2017; Chen and Ying, 2015; Kahle et al., 2008; Moschet et al., 2014; Zubrod et al., 2019). Besides, conazoles exert properties of persistency and bioaccumulative (Chen and Ying, 2015; Kahle et al., 2008). Conazole fungicides were designed to inhibit Erg11, a cytochrome P450 required for catalyzing demethylation in ergosterol biosynthesis (Turi and Loper, 1992), resulting in altered integrity of plasma membranes and increased content of fatty acids (Van den Bossche et al., 1983).

Additionally, the dysregulation of cholesterol biosynthesis and metabolism is a key event in conazole-induced liver tumors according to toxicological, genomic, proteomic and metabolomics studies (Hester et al., 2012; Nesnow, 2013; Ortiz et al., 2010). Conazoles have also been reported to have other adverse effects, such as cytotoxic effects, oxidative stress responses, DNA damage and possible carcinogenic effects (Bolcic-Tavcar and Vracko, 2012; EPA, 2004, 2011; Hester et al., 2012; Othmene et al., 2020; Yuzbasioglu et al., 2008). Conazoles have emerged as new environmental pollutants and posed potential risks to environmental organisms and humans, and have raised human health concerns in recent years (Gouin et al., 2012). However, the molecular mechanisms of toxicity that are required for conazoles to interact with cellular systems and affect several physiological functions are still unclear. Molecular fingerprints via functional genomic profiling could be more powerful to provide important clues for the mechanism of toxicity of conazoles.

Functional genomic single-gene knockout mutant screening can be

* Corresponding authors at: School of the Environment, Nanjing University, Nanjing, Jiangsu 210023, China.

E-mail addresses: xiaoniao8911@126.com (M. Guan), zhangxw@nju.edu.cn (X. Zhang).

used to identify strains with increased or decreased sensitivity in the presence of chemicals, providing an unbiased assessment and deep insights into the potential toxicity mechanism of chemicals (Alfatah et al., 2019; Guan and Zhang, 2017; Parsons et al., 2006; Xia et al., 2016); additionally, this method enables the identification of specific genes to establish a direct interaction between chemicals and adverse effects (Guan et al., 2018; Reczek et al., 2017). *Saccharomyces cerevisiae* (yeast) was chosen for this study to assess the adverse effects induced by conazoles due to its well-characterized features. Nearly half of the genes that yeast harbors are homologous to genes harbored by humans (Kachroo et al., 2015), and various genetic requirements for chemical tolerance identified in yeast have been confirmed in human cell lines (Gaytan and Vulpe, 2014). Moreover, compared with CRISPR-Cas9 based genome-scale engineering strategies in yeast (Schwartz et al., 2019) and mammalian cells (Xia et al., 2016), the strategy of gene knockout collection in the present study is convenient for validating the chemically-responsive genes because ~6000 individual mutants are stocked separately. Each single-gene knockout strain in our yeast mutant library has a unique molecular barcode with 20 bp DNA sequence tags, that make it possible to pool thousands of mutants and quantify the competitive growth of each gene at the same time, generating a chemical-genetic interaction profile (Alfatah et al., 2019; Giaever et al., 2002; Giaever et al., 2004; Nislow et al., 2016; Parsons et al., 2006).

To evaluate the molecular toxicity mechanism and explore the specific gene fingerprints of conazoles, five conazoles (Propiconazole (Pro), penconazole (Pen), tebuconazole (Teb), flusilazole (Flu) and epoxiconazole (Epo)) were selected for toxicological assessment using a yeast knockout mutant screening approach. The study aimed: (1) to assess the sensitivity fingerprints of genome-wide mutants to the five conazoles and to identify their responsive genes; (2) to compare the known toxicological mechanisms of conazoles with the molecular response by functional genomic analysis of yeast (Fig. 1); (3) to explore

the potential novel mechanisms of conazoles at the molecular level; and (4) to identify specific genes essential for conazole-induced toxicity.

2. Materials and methods

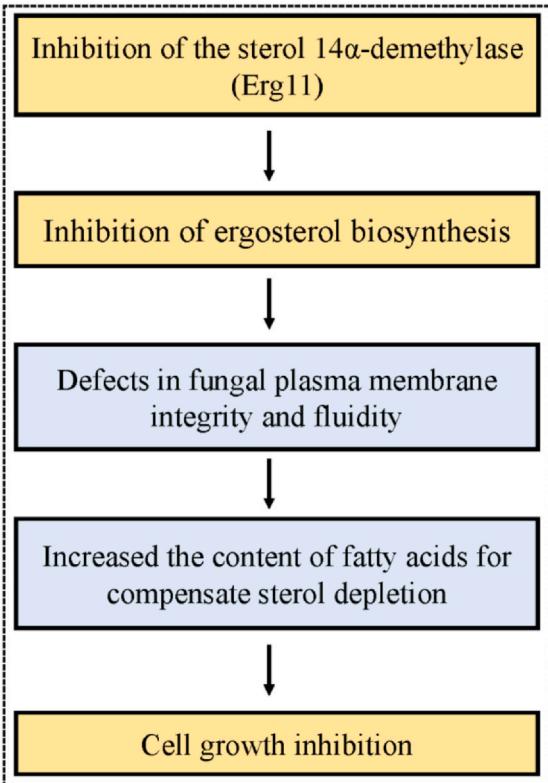
2.1. Chemicals

Five conazoles, including propiconazole, penconazole, tebuconazole, flusilazole and epoxiconazole, were purchased from Sigma Aldrich (St. Louis, MO, USA) (Table 1, Fig. S1). The stock solutions (100 mM) of the five conazoles were prepared in dimethyl sulfoxide (DMSO; St. Louis, MO, USA). All other test solutions were diluted in DMSO from the stock solutions.

2.2. Genome-wide yeast knockout strain library

The yeast knockout strains used for functional profiling were in the BY4743 background and contained ~6000 single-gene knockout strains, which were ~5000 homozygous knockout nonessential strains and ~1000 heterozygous knockout essential strains. The strains which from the *Saccharomyces* Genome Deletion Project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html) were purchased from Thermo Fisher SCIENTIFIC (<http://clones.thermofisher.com/cloneinfo.php?clone=yeast>, catalog number: 95400.BY4743) and were frozen at -80 °C in 96-well plates in liquid YPD medium containing 15% glycerol (Corning, NY, USA). The yeast strains were inoculated into another 96-well plate filled with 200 µL fresh liquid YPD medium (1% yeast extract, 2% peptone and 2% glucose) with 200 µg/mL G418 from the stock plate by using disposable replicators (Genetix, San Jose, CA, USA). Cells were incubated in liquid YPD for 72 h at 30 °C with shaking at 200 rpm. All mutant strains (~6000 single-gene knockout) were mixed as pooled strains with

Known mechanism by which conazoles induce cytotoxicity



Functional genomic analysis of yeast in this study

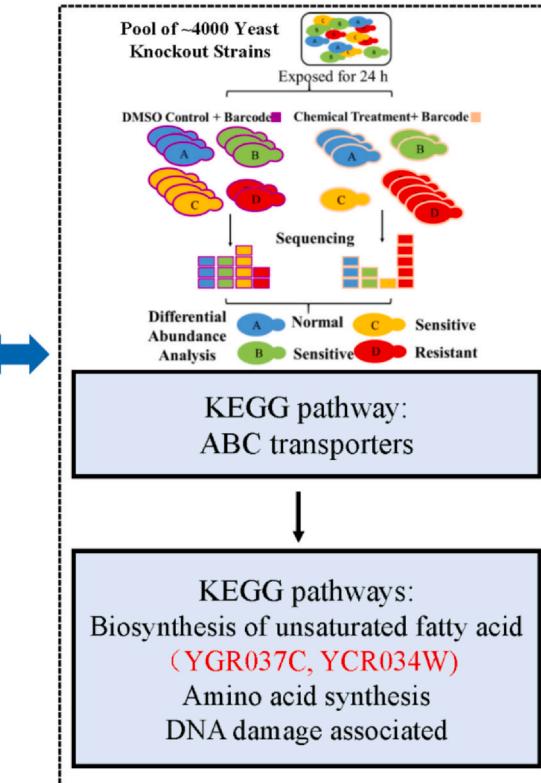


Fig. 1. Comparison of the known toxicological mechanism of conazoles with their molecular response by functional genomic analysis of yeast.

Table 1
Conazoles' information and 24 h yeast acute toxicities.

Chemical	Abbreviation	CAS	Maximum inhibition (%)	IC ₅₀ (μM)	IC ₂₀ (μM)	IC ₁₀ (μM)
Propiconazole	Pro	60,207–90-1	81.5	2.78 ± 0.28	0.22 ± 0.08	0.05 ± 0.013
Penconazole	Pen	66,246–88-6	78.94	20.53 ± 1.39	4.32 ± 0.103	1.91 ± 0.136
Tebuconazole	Teb	107,534–96-3	71.43	10.61 ± 0.33	1.05 ± 0.271	0.327 ± 0.18
Flusilazole	Flu	85,509–19-9	75.02	19.67 ± 0.57	3.17 ± 0.386	1.22 ± 0.209
Epoxiconazole	Epo	133,855–98-8	78.76	3.05 ± 0.31	0.118 ± 0.02	0.02 ± 0.004

approximately equal cell density and stored in a 50 mL centrifugal tube. The pooled which contain ~6000 mutants with a density of 1*10⁸ cells/mL in YPD medium with 7% DMSO were stored as pooled stock strains at -80 °C until use.

2.3. Cytotoxicity test

A cytotoxicity test of the five conazoles using a pool of ~6000 available mutants was conducted prior to the screening assay to determine IC₅₀, IC₂₀ and IC₁₀ for each conazole (with three replicates for each treatment and control). Pooled mutant strains were inoculated from pooled stock strains and cultured at 30 °C with shaking at 200 rpm overnight. Cells were diluted from the overnight culture to an OD₆₀₀ of 0.1 ± 0.02 with 10⁶ cells/mL using a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Cells were in the logarithmic phase, and each mutant had at least 200 cells before being exposed to seven diluted concentrations of each chemical. Cells were exposed to 10 μL chemical (1%) with a series of concentrations ($n = 3$) using a 24-well plate (Thermo Nunc) to reach a 1 mL culture system and incubated at 30 °C for 24 h with 200 rpm shaking. The OD₆₀₀, which represents the growth density of yeast cells, was measured at 24 h using a microplate reader. IC₅₀ (concentration causing 50% cell inhibition), IC₂₀ and IC₁₀ values with their standard deviation which represent the maximum, median and minimum concentration of chemicals for further exposure of yeast pooled strains were calculated using the probit equation with SPSS 16.0.

2.4. Functional profiling screening

The workflow of the whole analysis is shown in Fig. 2A. Ten microliters of chemicals (1%) with concentrations of IC₅₀, IC₂₀ and IC₁₀ or solvent control (DMSO) were added to the pooled cells under the same conditions as the cytotoxicity test using a 24-well plate and incubated at 30 °C for 24 h with 200 rpm shaking in triplicate. Following exposure to chemicals for a 24 h period, yeast cells were harvested after centrifugation, and genomic DNA was extracted by using the OMEGA E. Z. N. A. yeast DNA Kit (D3370-01). For amplification of the unique tags of each yeast deletion mutant strain, ~0.2 μg genomic DNA was combined with 250 μM deoxynucleoside triphosphate (dNTPs), 0.4 μM F primer and 0.4 μM R primer with specific barcodes, 5 × Phusion HF buffer (F518, Thermo), 2 U/μL Phusion hot start II DNA polymerase (Thermo) and nuclease-free water in a reaction volume of 20 μL. PCR conditions were as follows: 3 min at 94 °C; 28 cycles of 30 s at 94 °C; 30 s at 55 °C; and 30 s at 72 °C; followed by 3 min at 72 °C to ensure product elongation. DNA fragments of specified lengths were obtained by 2% agarose gel electrophoresis and gel purification with a Wizard SV Gel and PCR Clean-Up System (Promega). Target DNA of all samples was quantified using a QuBit fluorometer 2.0 (Thermo Fisher Scientific, Waltham, MA) and diluted to 100 pM, and then all samples were mixed as a pool for sequencing. Sequencing was performed on Ion Torrent Proton with Ion PI Template OT2 200 Kit v3 and Ion PI Sequencing 200 Kit v3 (Life technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.5. Statistical analysis of screening data

The raw FASTQ data from the Ion Torrent service were submitted to the edgeR package (McCarthy et al., 2012) in the statistical environment of R to match the 20 bp unique mutant barcode sequences of ~6000 mutants from 48 treatments (3 concentrations of the 5 conazoles and 1 DMSO control with 3 replications). Mutants with counts that were smaller than 10 in any of the DMSO controls were removed. The reads of each strain from each sample were normalized to balance the abundance of the total reads of each sample. The differential representations (p_{adj}) corrected for multiple testing of genes between the treatment and DMSO control, representing the relative abundance of cells with a single-gene knockout mutant, were calculated. Enriched genes were selected when $p_{adj} < 0.05$ using the DEseq2 package in R (Love et al., 2014). Enriched genes were responsive genes (sensitive genes or resistant genes) when the fold-change (treatment abundance/control abundance) of enriched genes was smaller than 1/1.5 or larger than 1.5 (Saquib et al., 2020). The sensitive genes (whose knockout enhanced the potential sensitivity to conazoles) were considered to be associated target genes involved in the repair of cellular damage by conazoles, while the resistant genes (whose knockout induced potential resistance to conazoles) were considered to be a loss of function of associated target genes (Zhang et al., 2018). This provided gene lists of the genes responsive to conazoles. The gene descriptions were found in the STRING website (<https://string-db.org/>) and Saccharomyces Genome Database (<https://www.yeastgenome.org/>). A fold-change heatmap of the multi-responsive genes (responsive at least in three treatments) of the five conazoles at three concentrations which aimed at visualization of the fingerprints of different conazoles was subjected to clustering by Pearson's Dissimilarity using Ward's Method.

Gene set enrichment analysis (GSEA) of Gene Ontology (GO) Biological Process (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to investigate the potential bioactivity of conazoles. The mutant strain was considered responsive to the chemical when it was responsive at least to one of three concentrations (IC₁₀, IC₂₀ and IC₅₀). So, the assessment of the mechanisms of toxic action was performed with GO BP and KEGG pathway GSEA via the R package clusterProfiler (Yu et al., 2012) by the combining the responsive genes from the three concentrations of each conazole. GO BP terms and KEGG pathways with p values less than 0.05 after multiple testing correction (p_{adj}) were considered significant.

An unsupervised approach (Fang et al., 2020; Xia et al., 2017) was performed to assess the top-ranked KEGG pathways based on fold-change values induced by conazoles. First, gene sets in each KEGG pathway were derived from the "org.Sc.sgd.db" package in R. Next, the fold-change of each pathway was calculated by the geometric mean of unified fold-changes (for sensitive genes: 1/fold-change; for resistant genes: fold-change) of the matched enriched genes ($p < 0.05$) induced by the maximum concentration of conazoles. Only KEGG pathways with at least one enriched gene were selected to represent the perturbation of pathways. At last, KEGG pathways were ranked by the fold-change values of each pathway (Fig. 2A.).

2.6. Comparing data with the existing chemical database

To compare the potential adverse effects provided by yeast

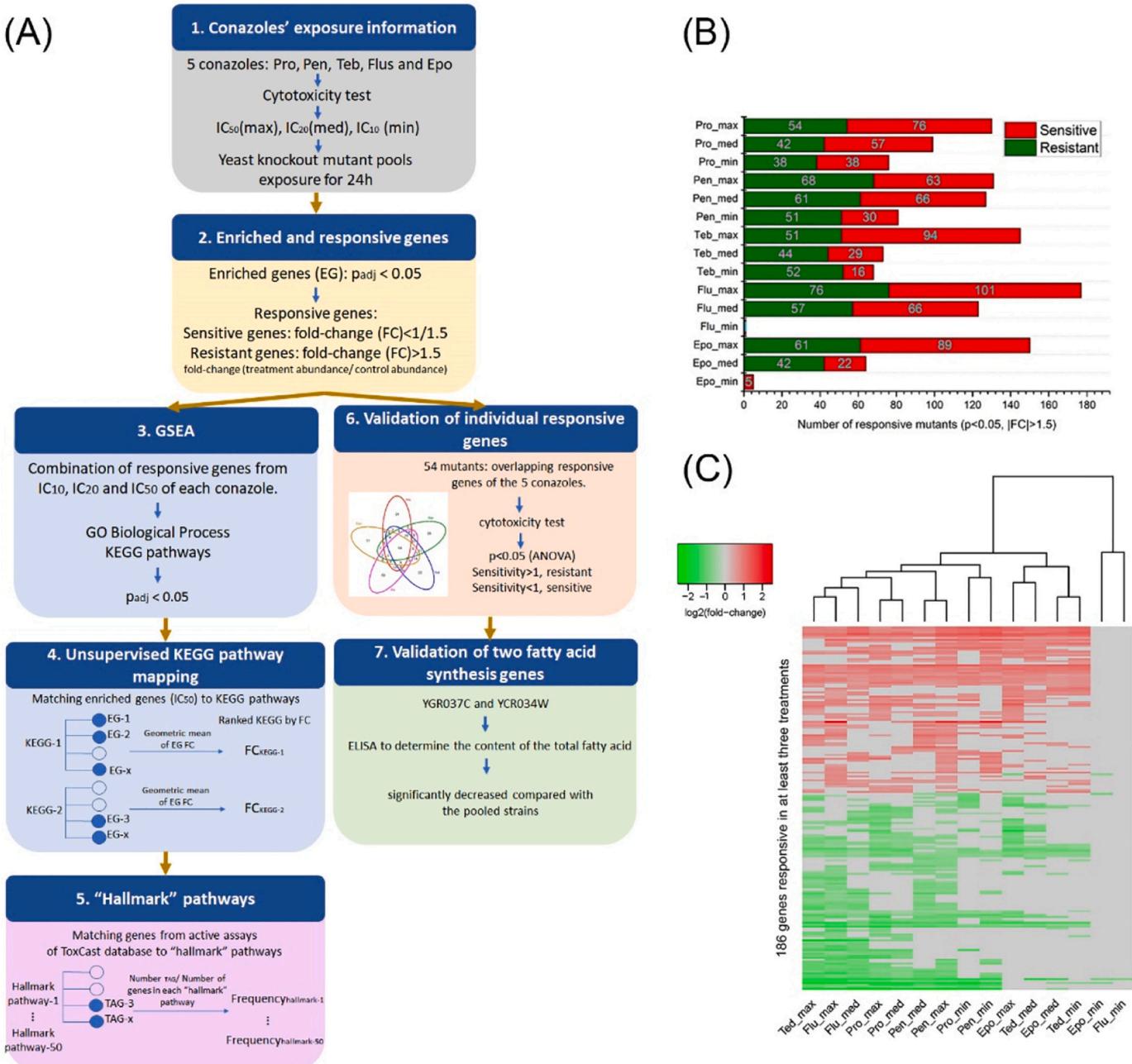


Fig. 2. (A) Workflow for evaluating molecular toxicity mechanism of conazoles using *Saccharomyces cerevisiae* functional genomic profiling. (B) Number of the responsive mutants induced by the five conazoles at three tested concentrations (max (IC₅₀), med (IC₂₀) and min (IC₁₀)). (C). Hierarchical clustering of the five conazole fungicides at three concentrations by the fold change (treatment abundance/ control abundance) of 186 multi-responsive genes (|fold-change| > 1.5 and $p < 0.05$ at least in three treatments).

functional genomic profiling with the existing chemical database, we investigated the U.S. Environmental Protection Agency ToxCast database (<https://comptox.epa.gov/dashboard>). 176 (19.9% of test assays), 70 (29.5%), 127 (17.0%), 237 (26.6%) and 81 (17.0%) active assays were selected from ToxCast by Pro, Pen, Teb, Flu and Epo, respectively. Then, the target active gene (TAG) sets from "gene-based" active assays for five conazoles were derived.

The gene sets from curated biological processes called "hallmark" pathways which contain 50 processes were downloaded from a previous study (Liberzon et al., 2015). The "hallmark" pathways which represented a well-defined and the widest range of cellular responses were used to evaluate the biological process of environmental pollutants recent years (Fang et al., 2020; Xia et al., 2017). The frequency of

each "hallmark" pathway was calculated as the number of active genes of each chemical divided by the number of genes in each "hallmark" pathway. Then the "hallmark" pathways were ranked by the frequency of each pathway. The most common top-ranking "hallmark" pathways of the five conazoles were associated with adverse effects induced by conazoles (Fig. 2A.).

2.7. Validation of individual responsive genes

A total of 54 mutants with overlapping genes among the five conazoles were selected for individual single-gene knockout mutant validation with the exposure concentration of IC₅₀. The mutants were selected individually from the stock plates and incubated overnight.

Then, the mutants were exposed to conazoles for 24 h before OD₆₀₀ was read, as with the cytotoxicity test (with three replicates for each treatment and control). Statistically significant differences ($p < 0.05$) between the responsive mutant (treatment_{OD600}/DMSO_{OD600}) and pooled strains (treatment_{OD600}/DMSO_{OD600}) were tested using analysis of variance (ANOVA) when the variance was homogeneous, and a Kruskal-Wallis test applied when the variance was heterogeneous. The sensitivity of the validated mutant was calculated using eqs. 1.

Sensitivity

$$\begin{aligned} &= \text{Responsive strains} \frac{\text{treatment OD600}}{\text{DMSO OD600}} \\ &/ \text{Pooled strains} \frac{\text{treatment OD600}}{\text{DMSO OD600}} \end{aligned} \quad (1)$$

The protein-protein interaction (PPI) network of 54 overlapped genes of five conazoles was evaluated by Search Tool for the Retrieval of Interacting Genes (STRING; <http://string-db.org>) (version 11.0) online database. Then the interaction networks of connected nodes with medium confidence of 0.4 were visualized by Cytoscape (version 3.7.2).

2.8. Validation of the YGR037C and YCR034W mutants by determination of the Total fatty acid content

Considering the results of top-ranked KEGG pathways and top-ranked hallmark pathways. And conazoles are also known to disturb the fatty acid synthesis process in other species (Hermsen et al., 2012; Hester and Nesnow, 2008; van Dardel et al., 2011; Wang et al., 2017). The knockout mutants YGR037C and YCR034W, which encode fatty acid synthesis proteins and validated to be resistant to conazoles in this study were chosen to test the role in conazole-induced toxicity.

The knockout mutants YGR037C and YCR034W and pooled strains were individually exposed to the IC₅₀ of the five conazoles in triplicate for 24 h at 37 °C with 200 rpm shaking. Yeast cells were lysed with lyticase for 30 min after centrifugation, and the medium was discarded. The total fatty acid content between cells treated with conazoles and cells treated with DMSO as the control was measured by an enzyme-linked immunosorbent assay (ELISA) kit (ml79612, Shanghai Enzyme-linked Biotechnology Co., Ltd.) using the mutants YGR037C and YCR034W and pooled strains.

3. Results and discussion

3.1. Inhibition of yeast cells

The maximum test concentration (100 μM) for five conazoles were soluble in the test system medium. The five tested conazoles were cytotoxic at the maximum concentration (100 μM), with a maximum inhibition of 81.5%, 78.94%, 71.43%, 75.02 and 78.76% for Pro, Pen, Teb, Flu and Epo, respectively (Fig. S2). The IC₅₀ values of the five conazoles were 2.78, 20.53, 10.61, 19.67 and 3.05 μM for Pro, Pen, Teb, Flu and Epo, respectively. The IC₂₀ and IC₁₀ values were shown in Table 1. The acute toxicity of Pro and Epo was greater than that of Pen, Teb and Flu.

3.2. Functional profiling screening

The normalized counts of each mutant strain from each sample (five conazoles from 3 concentrations with three replicates) were shown in Table S1. The number of responsive genes (within three test concentrations with $p_{adj} < 0.05$ and fold-change $< 1/1.5$ or > 1.5) were 169 (4.49%), 176 (4.67%), 198 (5.26%), 218 (5.79%) and 173 (4.59%), with 99, 86, 112, 115 and 98 sensitive genes and 70, 90, 86, 103 and 75 resistant genes for Pro, Pen, Teb, Flu and Epo, respectively (Table S3 and Fig. S3). The fold-change range of responsive genes across the five conazoles at the three concentrations was 0.17–4.94 (Fig. 2C). The

number of sensitive strains and resistant strains was similar for the five conazoles (Fig. 2B). The five conazoles at the three tested concentrations (except for Epo_min and Flu_min) tended to have relatively similar responsive gene fingerprints (Fig. 2C). The number of responsive genes by exposure to each conazole was proportional to the concentrations of the individual conazole (Fig. 2B). Genes responsive at lower or moderate concentrations were almost responsive at greater concentrations (Fig. S3). The genes set of GSEA were selected from genes responsive in at least one concentration of each chemical (Guan et al., 2016). Fold-change values of enriched genes with the maximum concentration were selected for further analysis of unsupervised KEGG pathway mapping.

3.3. Mechanisms of toxic action by GSEA

Disturbances in protein synthesis may serve as a potential molecular mechanism of the five conazoles, as revealed by the enriched GO BP terms and KEGG pathways. The GO BP term commonly perturbed by five conazoles was “cytoplasmic translation” (GO: 0002181). The KEGG pathway commonly perturbed by the five conazoles was the “Ribosome” pathway (sce03010). Moreover, four GO BP terms, “translation” (GO: 0006412), “peptide biosynthetic process” (GO: 0043043), “peptide metabolic process” (GO: 0006518), and “amide biosynthetic process” (GO: 0043604), and the KEGG pathway “phenylalanine, tyrosine and tryptophan biosynthesis” (sce00400), were the pathways commonly induced by three conazoles (Pro, Teb and Epo) (detailed pathways and corresponding p_{adj} , see Fig. 3). These pathways were associated with the synthesis of amino acids. In addition, the synthesis of amino acids was also affected by Epo in a previous study using zebrafish metabolomics profile analysis (Wang et al., 2017). It was also reported that conazole (3-amino-1,2,4-triazole) affected protein biosynthesis by using yeast gene deletion mutant screening (Alamgir et al., 2010). Therefore, the five conazoles influenced protein synthesis in the cell.

3.4. KEGG pathway ranking by enriched genes

There were 34 (34.3% of all yeast KEGG pathways), 36 (36.4%), 40 (40.4%), 48 (48.5%) and 42 (42.4%) KEGG pathways affected by at least one enriched gene in the corresponding pathways for Pro, Pen, Teb, Flu and Epo, respectively (Fig. 4). The most ranked KEGG pathways for the five conazoles were “biosynthesis of unsaturated fatty acids”, “phenylalanine, tyrosine and tryptophan biosynthesis”, “sulfur relay system” and “ribosome”. The pathways “Phenylalanine, tyrosine and tryptophan biosynthesis” and “ribosome” were selected from both the GSEA and top-ranking KEGG pathway analysis. Lipid metabolism or fatty acid metabolism was also disturbed in other organisms, such as zebrafish and rats, by conazoles in previous studies (Hermsen et al., 2012; Hester and Nesnow, 2008; van Dardel et al., 2011; Wang et al., 2017). The “Sulfur relay system” pathway is a novel mechanism that conazoles induced in this study. The “Sulfur relay system” pathway is for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions (Noma et al., 2009). This may imply that conazoles influence cellular sulfur transfer. The KEGG pathway of “ATP binding cassette (ABC) transporters”, which is in the category of membrane transport induced by conazoles (Pen and Flu), corresponded to the findings of an earlier study showing that conazoles influence the transport of cell membranes (Liu et al., 2010). Earlier study demonstrated that azoles were substrates of ATP-binding cassette superfamily (ABC) transporters (Sanglard and Coste, 2016). Moreover, the KEGG pathways of “DNA replication”, “homologous recombination”, “base excision repair”, “mismatch repair” and “nucleotide excision repair” induced by Teb and Flu indicated that conazoles induced DNA repair, which might elucidate the mechanism by which conazoles are potential carcinogens (Bolcic-Tavcar and Vrakko, 2012). This is consistent with previous studies that tebuconazole would induce DNA damage in kidney of male

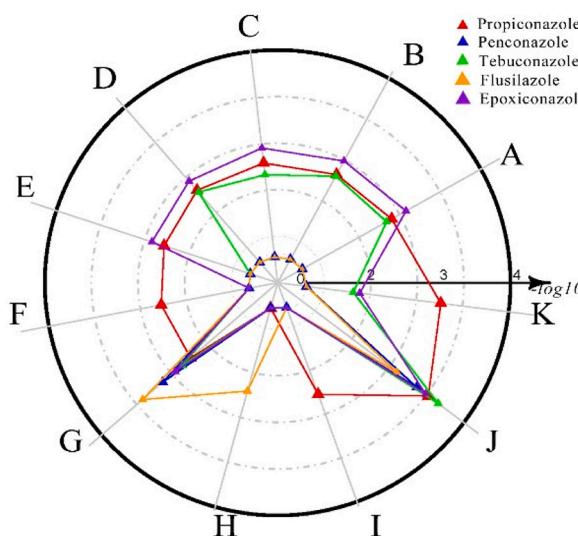


Fig. 3. GO BP terms and KEGG pathways which were significantly enriched (adjusted p -value < 0.05) by GSEA of the responsive genes of the five conazoles indicated by different colors.

Wistar rats (Othmene et al., 2020).

3.5. Comparison with the existing chemical database

After calculation of the frequency of “hallmark” pathways from the target active gene in ToxCast database, the frequency of bile acid metabolism pathway in the “hallmark” pathway ranked high for all five conazoles (Pro, Pen, Teb, Flu and Epo) (Fig. S4). Bile acid is crucial for lipid synthesis and metabolism. This demonstrated that fatty acid synthesis and metabolism have a vital role in conazole-induced toxicity.

Symbols	GO or KEGG id	Pathways
A	GO:0006412	Translation
B	GO:0043043	Peptide biosynthetic process
C	GO:0006518	Peptide metabolic process
D	GO:0043604	Amide biosynthetic process
E	GO:0043603	Cellular amide metabolic process
F	GO:1901566	Organonitrogen compound biosynthetic process
G	GO:0002181	Cytoplasmic translation
H	GO:0042274	Ribosomal small subunit biogenesis
I	GO:0009073	Aromatic amino acid family biosynthetic process
J	sce03010	Ribosome
K	sce00400	Phenylalanine, tyrosine and tryptophan biosynthesis

3.6. Validation of responsive strains

Out of 54 overlapped responsive genes of five test conazoles (Fig. S3F), 49, 47, 51, 48 and 49 genes were validated correctly for Pro, Pen, Teb, Flu and Epo, with overall accuracies of 90.74%, 87.04%, 94.44%, 88.89% and 90.74%, respectively. The sensitivity of most of the 54 genes induced by the five conazoles was very similar (Fig. 5). This was consistent with a previous study showing that conazoles had similar gene expression profiles and similar mechanisms of toxic actions (Hermsen et al., 2012; Martin et al., 2007).

The PPI network consisted of 30 nodes and 41 edges, including 11

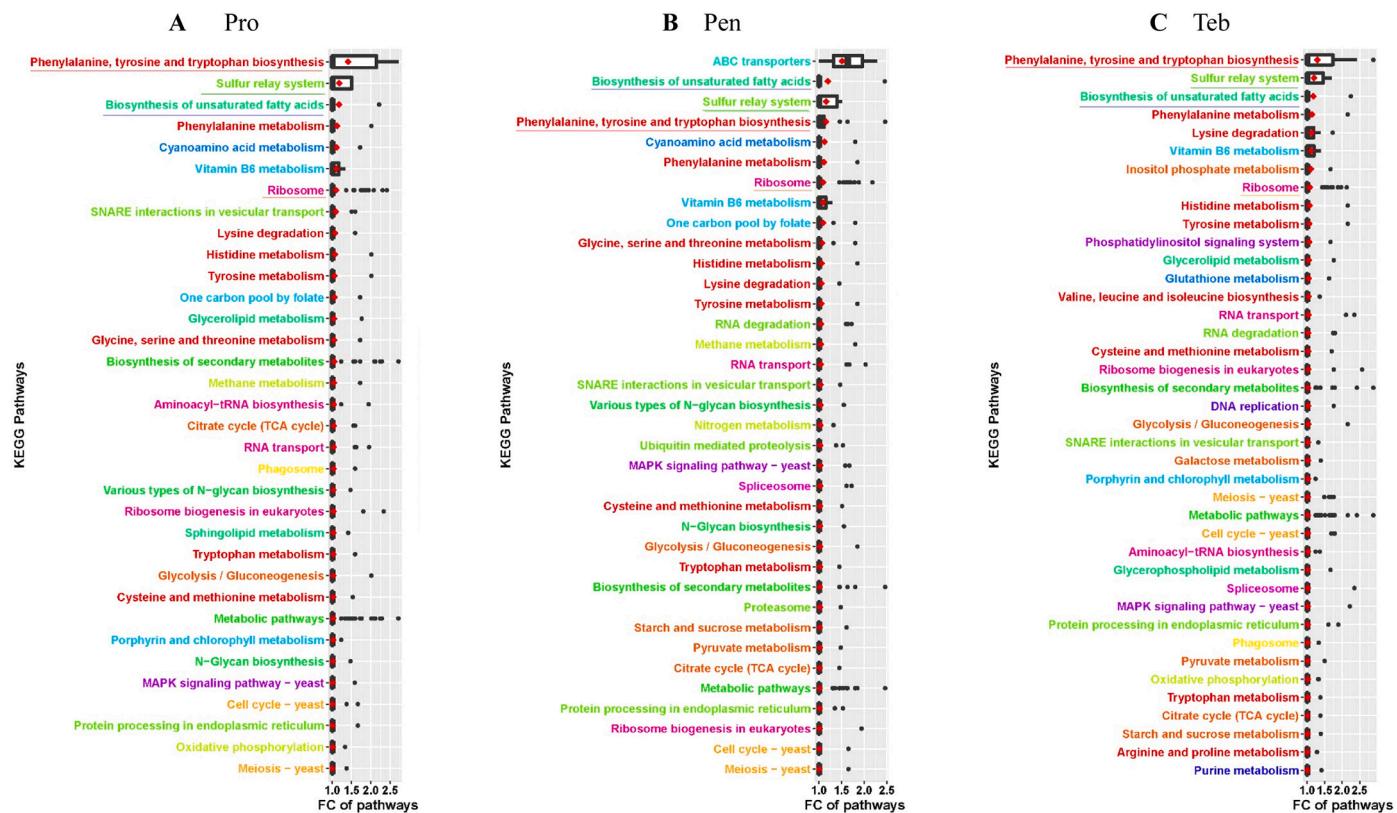


Fig. 4. Ranked KEGG pathways based on fold-change values of the corresponding enriched genes from IC_{50} concentration of the five conazoles.

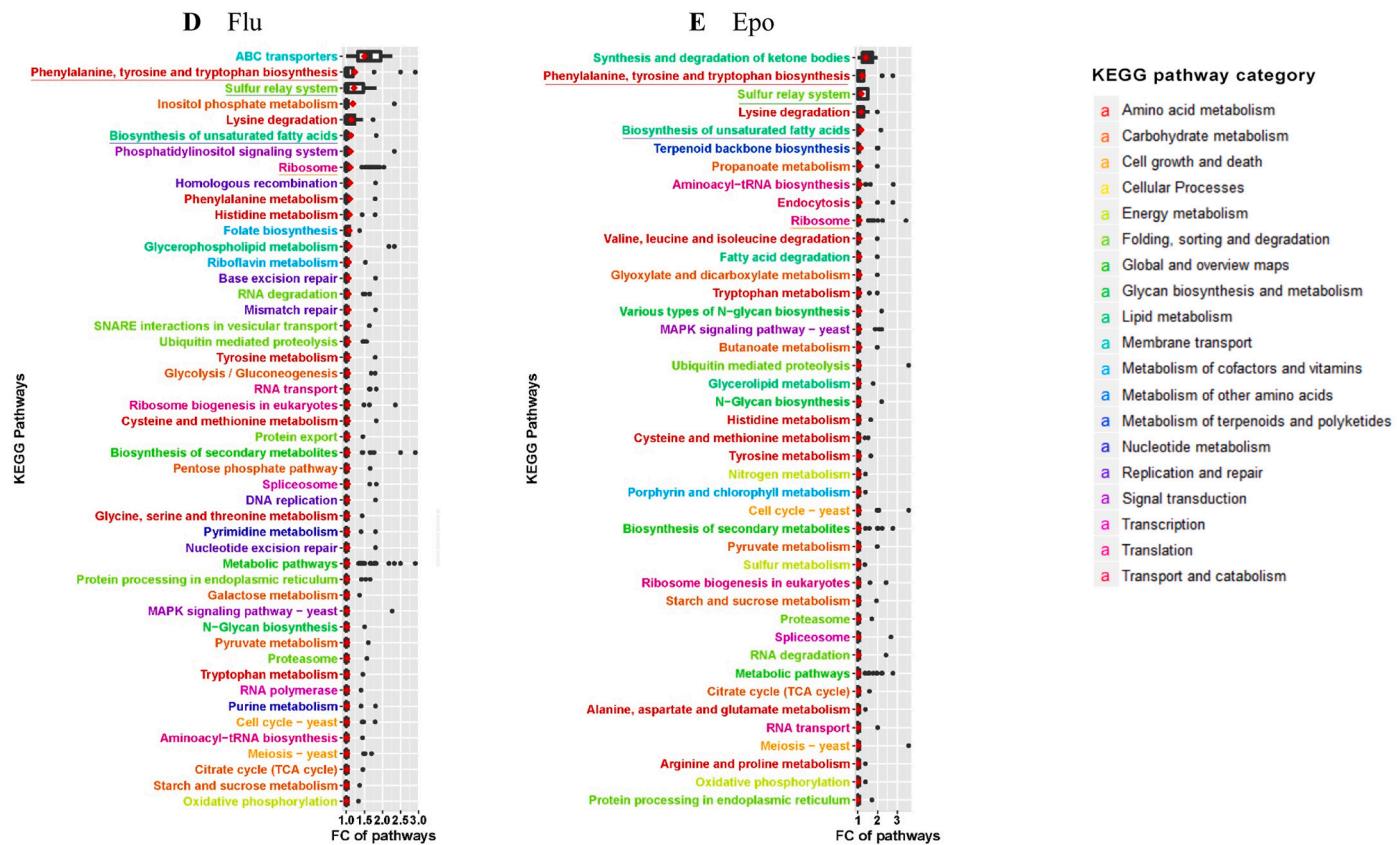


Fig. 4. (continued)

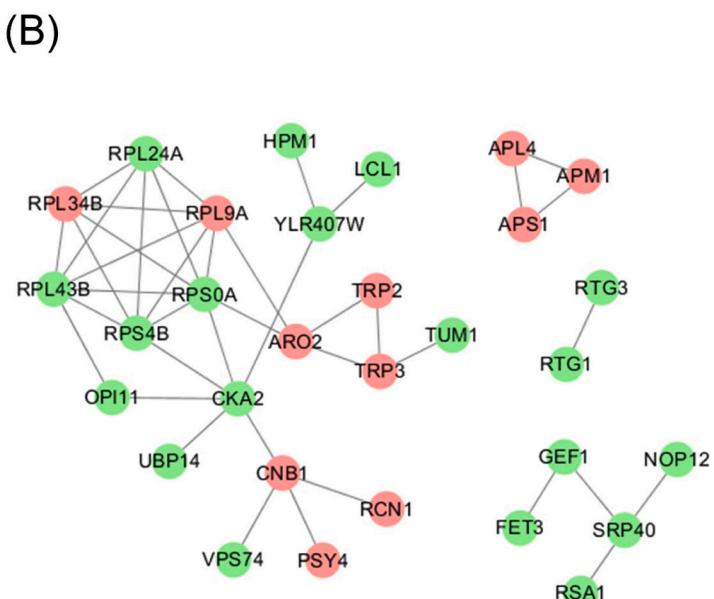
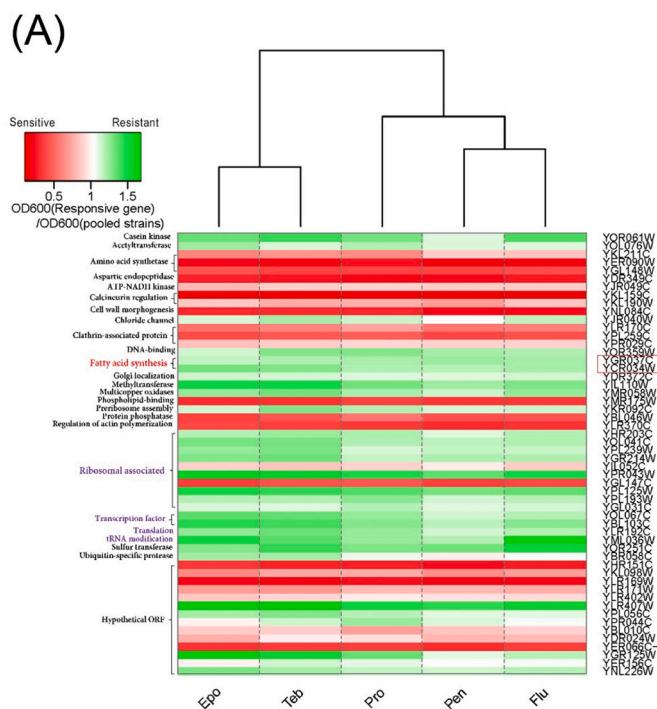


Fig. 5. (A) Sensitivity of the 54 responsive genes of the five conazoles. **(B)** The PPI network of overlapped responsive genes of five test conazoles was constructed using STRING and Cytoscape. The nodes meant proteins; the edges meant the interaction of proteins. Sensitive genes are marked in light red; resistant genes are marked in light green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

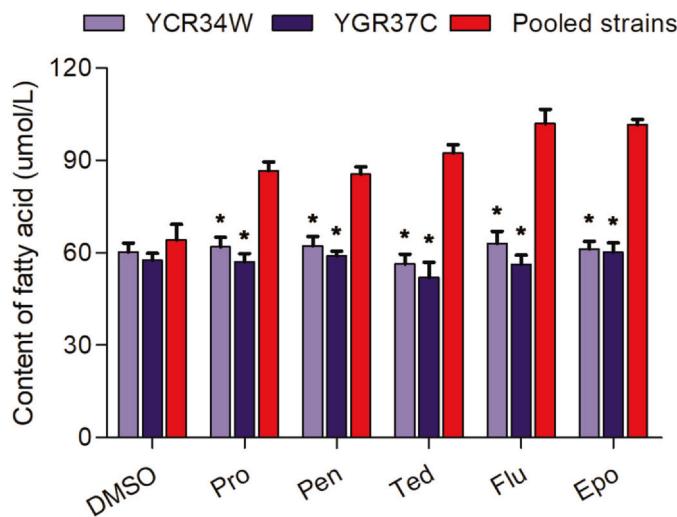


Fig. 6. Content of fatty acids of the YCR034W and YGR037C mutant strains compared with that of the pooled strains. The mutants YCR034W and YGR037C showed significantly decreased fatty acid contents compared with pooled strains exposed to the five conazoles by statistical analysis * $p < 0.01$.

sensitive genes and 19 resistant genes. 24 genes were disconnected nodes and were not contained into the PPI network (Fig. 5B). Two genes (YGR037C and YCR034W), which were associated with fatty acid synthesis, became resistant to conazole exposure, indicating that fatty acid synthesis was one of the targets of conazole-induced toxicity (Hermsen et al., 2012; Wang et al., 2017). YGR037C (ACB1) is an acyl-CoA-binding protein that is a long-chain fatty acid transporter. YCR034W (ELO2) encodes a fatty acid elongase involved in sphingolipid biosynthesis and in the KEGG pathway of “biosynthesis of unsaturated fatty acids”. The detailed gene function descriptions of the 54 responsive genes can be found in Table S3. Out of 54 genes, 10 (YHR203C, YOL041C, YPL239W, YGR214W, YIL052C, YPR043W, YGL147C, YPL125W, YPL193W and YGL031C), 2 (YOL067C and YBL103C), 1 (YLR192C) and 1 (YML036W) genes were associated with ribosome, transcriptional factor, translation and tRNA modification, respectively. The functions of these 14 genes were all related to protein synthesis. There were 12 genes with unknown function, and 2 (YLR407W and YPL056C) out of 12 genes were reported to be resistant to conazole (fluconazole) (Anderson et al., 2003), which was in accordance with our results. The validation accuracy was above 87% for all five conazoles, demonstrating that the screening method through sequencing was a powerful approach for exploring the specific genes involved in key mechanisms of chemical-induced toxicity.

3.7. YGR037C and YCR034W played key roles in conazole induced toxicity

The mutants YCR034W and YGR037C showed significantly decreased fatty acid contents compared with the pooled strains exposed to the five conazoles by statistical analysis * $p < 0.01$ (Fig. 6). The results validated that YGR037C and YCR034W played key roles in conazole-induced toxicity. YGR037C (ACB1) has homologous genes in many organisms, such as humans (ACBD 4, ACBD5, ACBD7 and DBI). YCR034W (ELO2) also has homologue genes in humans (ELOVL1, ELOVL2, ELOVL3, ELOVL4, ELOVL5, ELOVL6 and ELOVL7). The functions of YGR037C and YCR034W are similar even in other organisms, which confirmed that conazoles disturbed lipid metabolism, fatty acid metabolism and bile acid metabolism in other organisms, such as zebrafish, rats, and humans (Hermsen et al., 2012; Hester and Nesnow, 2008; van Dartel et al., 2011; Wang et al., 2017). Previous study also demonstrated that conazole treatment led to a statistically significant increase in cellular triglycerides in human HepaRG cells after 24 and

72 h by using the fluorescence-based AdipoRed assay (Luckert et al., 2018). Lipid accumulation were one of key events of conazole induced liver steatosis (adverse outcome) (Luckert et al., 2018).

4. Conclusions

The five tested conazoles had relatively similar responsive gene patterns and molecular toxicity mechanisms. At the pathway level, protein synthesis was the potential molecular mechanism of conazoles revealed by the enriched GO BP terms and KEGG pathways. Moreover, the KEGG pathway “ABC transporters” ranked the highest and validated the findings of a previous study indicating that conazoles influence plasma membrane integrity. Some DNA damage-associated pathways provide insight into the potential carcinogenic properties of conazoles. Fatty acid synthesis and metabolism played vital roles in conazole-induced toxicity in cells, especially two genes, YGR037C (ACB1, acyl-CoA-binding protein) and YCR034W (ELO2, fatty acid elongase). The yeast mutant screening approach employed here provides important clues for further deep studies of the mechanism of toxicity of environmental pollutant conazole fungicides.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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