J. Gen. Appl. Microbiol., **64**, 84–89 (2018)
doi 10.2323/jgam.2017.08.002

jGAM

Full Paper

γ-aminobutyric acid accumulation enhances the cell growth of *Candida glycerinogenes* under hyperosmotic conditions

(Received June 11, 2017; Accepted August 13, 2017; J-STAGE Advance publication date: March 12, 2018)

Hao Ji,[1,2] Xinyao Lu,[1,2] Hong Zong,[1,2] and Bin Zhuge[1,2,*]

[1] *The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China*
[2] *The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China*

γ-aminobutyric acid (GABA) is an important non-protein amino acid involved in the response to various environmental stresses in plant cells. The objectives of this study was to test the hypothesis that intracellular accumulation of GABA improves osmotic tolerance in the unconventional yeast *Candida glycerinogenes*. In *C. glycerinogenes*, the expression of *UGA4* encoding GABA-specific permease is highly induced by hyperosmotic stress. Exogenous GABA application enhanced intracellular GABA accumulation and promoted cell growth under hyperosmotic conditions. Overexpression of the glutamate decarboxylase gene *GAD1* resulted in an increased intracellular GABA and improvement in cell growth under hyperosmotic conditions. These results indicated that improving intracellular GABA accumulation of *C. glycerinogenes*, either through exogenous application or cellular synthesis, is available for improving the tolerance to hyperosmotic stress. We demonstrate that GABA accumulation plays an important role in osmotic stress resistance of the unconventional yeast *C. glycerinogenes*.

Key Words: *Candida glycerinogenes*; GABA-specific permease; γ-aminobutyric acid; glutamate decarboxylase; hyperosmotic stress

Introduction

The nonconventional yeast *Candida glycerinogenes* is proposed as a potential biotechnological host due to several convenient properties, such as its high tolerance to osmotic stress, low pH, and elevated temperature. It has been successfully used for glycerol production in a commercial scale because of a high yield (more than 120 g/L) and consumption rate of glucose (64.5%) (Zhuge et al., 2001). Since *C. glycerinogenes* produces large amounts of glycerol, its major strategy for survival under hyperosmotic conditions seems to be to accumulate glycerol as a compatible osmolyte to maintain the water balance and reestablish the turgor of the cells (Saito and Posas, 2012). In the model yeast *Saccharomyces cerevisiae*, glycerol serves as the major compatible osmolyte during the osmotic adaptation, and the enzymes glycerol-3-phpsphate dehydrogenase (Gpd1) and glycerol-3-phosphatases (Gpp1 and Gpp2) responsible for the synthesis of glycerol, are upregulated upon osmotic stress (Parmar et al., 2011; Remize et al., 2003; Rep et al., 1999). In addition, some other mechanisms and molecules involved in the osmoregulation have also been discovered (Saxena and Sitaraman, 2016).

Besides glycerol, a number of compatible osmolytes, such as carbohydrates, amino acids and betaines, were reported to contribute to the osmoadaptation (Saito and Posas, 2012). Proline, a major osmolyte in plants that accumulates in response to osmotic stress, also acts as an osmoprotectant in various yeasts (Matsuura and Takagi, 2005; Takagi, 2008; Xu et al., 2010). The global gene expression analysis of *S. cerevisiae* revealed that the expression of *PUT4* encoding a high-affinity proline permease is upregulated under hyperosmotic stress conditions (Rep, 2000), and the proline levels are due primarily to the increase in proline uptake from a nutrient medium (Kaino and Takagi, 2008). Xu et al. (2011) demonstrated that arginine was a better osmoprotectant than proline in terms

*Corresponding author: Bin Zhuge, The Key Laboratory of Carbohydrate Chemistry and Biotechnology; and The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China.
Tel (Fax): +86-0510-85918150 E-mail: bzhuge@126.com

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

of promoting cell growth under hyperosmotic conditions in *Candida glabrata*. The growth of *C. glabrata* was enhanced upon either the application of arginine, or the overexpression of the key enzymes of the arginine synthesis pathway (Guan et al., 2017; Xu et al., 2011).

Recently, γ-aminobutyric acid (GABA) was found to exhibit important physiological functions involved in stress tolerance (Karatzas et al., 2010; Wang et al., 2017). Earlier studies showed that GABA levels of some plant cells increased when exposed to different stress conditions (Kinnersley and Turano, 2000). In particular, application of γ-aminobutyric acid (GABA) induces an enhanced defense response towards osmotic stress. Exogenous GABA application could alleviate damage to membrane, result in an increase in proline and soluble sugar content, and reduce water loss during osmotic stress. These effects reduce the damage to the photosynthetic system and improve the chlorophyll fluorescence parameters of plant cells (Vijayakumari and Puthur, 2015; Wang et al., 2017). Although there is no direct evidence that the intracellular GABA affects the osmotic tolerance of yeast, a metabolomics analysis in *S. cerevisiae* revealed the crucial role of a GABA shunt for the resistance to multiple inhibitors such as furfural, acetic acid and phenol (Wang et al., 2013). A prior study also showed that the GABA-producing enzyme glutamate decarboxylase (GAD) was required for the normal oxidative stress tolerance. Genetic elimination of *GAD1* induced a hypersensitivity to oxidants, and increasing the gene dosage of *GAD1* produced an increased tolerance to the oxidative agents H_2O_2 and diamide (Coleman et al., 2001). Further study suggested that a GABA shunt pathway involving GAD, GABA aminotransferase, and succinate semialdehyde dehydrogenase, plays a crucial role in restricting ROI production (Cao et al., 2013). In the present study, the intracellular accumulation of GABA is hypothesized to improve the osmotic tolerance of the non-conventional yeast *C. glycerinogenes*, as it does in plant cells. We demonstrated for the first time that accumulation of intracellular GABA under hyperosmotic stress could be an alternative way to improve the osmotic tolerance of *C. glycerinogenes*.

Materials and Methods

Strains, media and growth conditions. *C. glycerinogenes* UA5 and the *GPD1*-deleting mutant S23 were cultured at 37°C in YPD medium (20 g glucose l^{-1}, 20 g peptone l^{-1}, and 10 g yeast extract l^{-1}) or synthetic dextrose (SD) medium (6.7 g yeast nitrogen base without amino acids l^{-1}, and 20 g glucose l^{-1}). For a growth test, 0.5 M or 1 M NaCl was added into the medium supplemented with, or without, 0.5 mM GABA. *Escherichia coli* JM109 was cultured in LB medium (10 peptone l^{-1}, 5 g yeast extract l^{-1}, and 10 g NaCl l^{-1}) containing 100 μg ampicillin ml^{-1} at 37°C for plasmid propagation.

Construction of GAD1 overexpression yeast strain. For an overexpressing of the glutamate decarboxylase gene (*GAD1*) in *C. glycerinogenes* UA5, the integration expression vector pURGAPU was used in this study (Ji et al., 2017). *GAD1* was amplified using primers GAD-F and GAD-R and then inserted into pURGAPU after being digested with the restriction enzymes *BamH* I and *Kpn* I. The recombinant plasmid was then linearized using the restriction enzyme *Sac* I for transformation. The resulting recombinant strain was designated as UA5-GAD1.

Table 1. Primers used for cloning *GAD1* and qRT-PCR.

Primer name	Sequence (5′ to 3′)
GAD-Bam	CGCGGATCCATGACACTTTCCAGCCATGT
GAD-Kpn	CTTGGTACCTTAACAAATAACTTTTGCATTTGAG
RT-ACTr	CATTGTTATGTCTGGTGGTA
RT-ACTf	TTGGAAGGTGGATAGAGAT
RT-GPDr	GTCCATGTATGTTGTCCTCC
RT-GPDf	ATTGTGAGAGGTGGCAAGGA
RT-GADr	CTTGGAAGAATCTGGTTAC
RT-GADf	GTCGTGCTCATCTATAATC
RT-UGAr	CATTAGCCTCACCTATTAG
RT-UGAf	AATCGTCTCATCAATTCC
RT-GAPr	ATCATACAACGAGAAGGA
RT-GAPf	AATATCAGTGAGGTTAGGA
RT-PUTr	CTGATGGTAATACTGGAA
RT-PUTf	GTAGATTGACTCTTGGAT

Underlined nucleotide sequences represent restriction sites introduced into the primer sequence.

DNA transformation of C. glycerinogenes. *C. glycerinogenes* UA5 was pre-cultured in YPD for 16 h and then inoculated into fresh medium at 30°C for 4 h. Cells were collected by centrifugation at 5,000 *g* for 5 min, washed twice with sterile water, and then resuspended in 360 μl of transformation buffer containing 240 μl of 50% PEG3350 (w/v), 36 μl of 1 M lithium acetate, 10 μl of 5 g/l ssDNA, deionized water and approximately 5 μg linearized plasmids DNA. The suspension was mixed thoroughly and then subjected to heat shock for 60 min at 42°C. Cells were then collected by centrifugation at 5,000 *g* for 5 min, washed twice with sterile water and then spread on the selective plates.

RNA extractions and real time PCR. *C. glycerinogenes* UA5 was grown up to the exponential phase in YPD medium at 30°C for 16 h, and then collected for stress treatment. After incubation, the cells were collected by centrifugation for total RNA extraction and the synthesis of cDNA, as previously described (Ji et al., 2016). Real-time PCR was performed in a Bio-Rad CFX96 Real-Time PCR system. Each reaction mixture contained cDNA (10 ng), 2 × UltraSYBR Mixture with ROX (CWbiotech™, 25 μl), 10 μM forward and reverse primers (1 μl), and RNase-Free water (up to 50 μl). *ACT1* was used as an endogenous reference with the primers ACTr and ACTf. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The gene sequences were obtained from the genome sequence of *Pichia kudriavzevii* (synonym for *C. glycerinogenes*, GCA_001983325.1) present in GenBank. All the primers are listed in Table 1.

Extraction of intracellular GABA. Culture samples were harvested by centrifugation at 8,000 *g* and washed twice with 0.9% NaCl and then suspended in 1 ml distilled water. The microcentrifuge tube containing suspended cells was transferred to a boiling water bath, and intracellular GABA was extracted by boiling for 10 min (Takagi et al.,

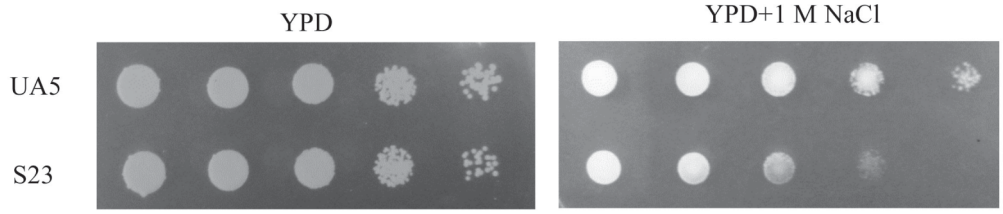

Fig. 1. Growth phenotype of *C. glycerinogenes* under hyperosmotic stress.

Yeast strains were grown in YPD medium to mid log phase, and the cells were collected after centrifugation at 6,000 *g*, and then serially diluted, spotted on YPD plates supplemented with, or without, 1 M NaCl. The plates were incubated at 37°C for 2 d and then photographed.

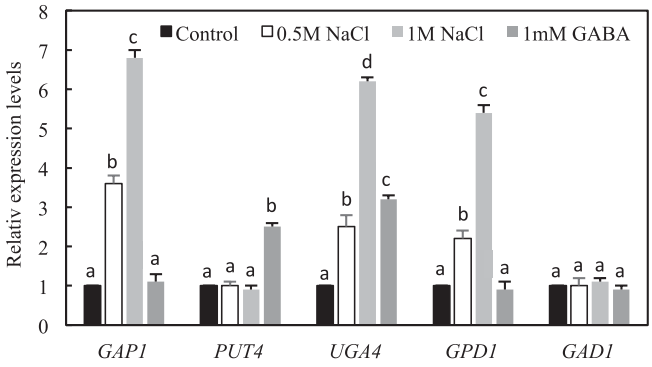

Fig. 2. Relative expression levels of GABA transport and synthesis related genes in *C. glycerinogenes*.

Yeast cells were grown to mid log phase in SD medium for 16 h at 37°C and then exposed to NaCl or GABA for 30 min. The relative expression levels were represented as the multiple of the expression without treatment. The value of each gene was normalized to the value of the internal control gene *ACT1*. All the values were the means of three biological replicates ± standard deviation. Letters indicate significant differences ($p < 0.05$, Duncan's multiple range test).

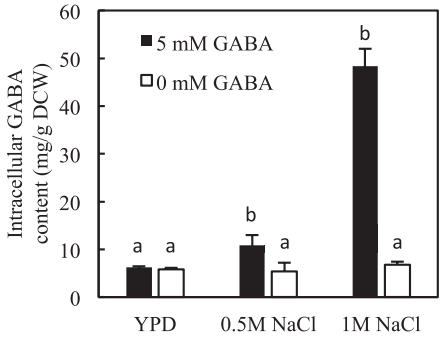

Fig. 3. Intracellular GABA accumulation of *C. glycerinogenes*.

The *C. glycerinogenes* cells were cultured in the SD medium under osmotic stress conditions with, or without, 5 mM GABA application for 16 h at 37°C. All the values are the means of three biological replicates ± standard deviation. Letters indicate significant differences ($p < 0.05$, Duncan's multiple range test).

2016). After centrifugation for 5 min at 10,000 × *g*, the supernatant was collected for measurement of intracellular GABA content.

Analytical methods. Biomass was measured as optical density at 600 nm after appropriate dilutions. The value of OD600 was calibrated to the drying cell weight (DCW) using the equation 1 OD600 = 0.33 g DCW l^{-1}. GABA concentration was quantified with a specific amino acid analyzer performed by the method as Xu et al. (2010) described.

Results and Discussion

Cell growth of C. glycerinogenes under hyperosmotic stress

C. glycerinogenes UA5 and the *GPD1* deletion mutant S23 were cultured in the YPD medium supplemented with, or without, 1 M NaCl to examine the effect of hyperosmotic stress on cell growth. Since *C. glycerinogenes* possesses only a single copy of the *GPD1* gene in the genomic DNA (Chen et al., 2008), the glycerol production of the *GPD1* deletion mutant S23 was eliminated (data not shown). Under non-stress conditions, there was no significant difference in cell growth between UA5 and S23; however, a slightly impaired growth of the *GPD1* deletion mutant S23 was observed in the presence of 1 M NaCl (Fig. 1), which highlighted the conservative osmoprotectant role of glycerol (Saxena and Sitaraman, 2016; Siderius et al., 2000). In *S. cerevisiae*, producing and accumulating glycerol is the major strategy for survival under hyperosmotic stress, although there are a number of mechanisms that contribute differently to the osmoadaptation (Hohmann, 2002; Saxena and Sitaraman, 2016). In the present study, we consider that exogenous amino acid in the YPD medium might enter the cells through amino acid permease to operate the osmotic tolerance of *C. glycerinogenes* S23, and the intracellular accumulation of GABA is hypothesized to improve osmotic tolerance in *C. glycerinogenes* cells, as it does in plant cells (Bouche and Fromm, 2004; Kinnersley and Turano, 2000).

Expression of GABA permease genes are highly induced by hyperosmotic stress

To clarify the potential functions of GABA in *C. glycerinogenes*, the expression patterns of general amino acid permease gene *GAP1*, proline-specific permease *PUT4* and GABA-specific permease *UGA4* involving in GABA transport, as well as the glutamate decarboxylase gene *GAD1* involving in GABA synthesis were analyzed by using qRT-PCR. Transcription analyze carried out with the cells incubated for 30 min in SD medium in the presence of NaCl revealed that *PUT4* was constitutively expressed in *C. glycerinogenes*, unlike that in *S. cerevisiae* (Rep, 2000). In contrast, the expression of *GAP1* and

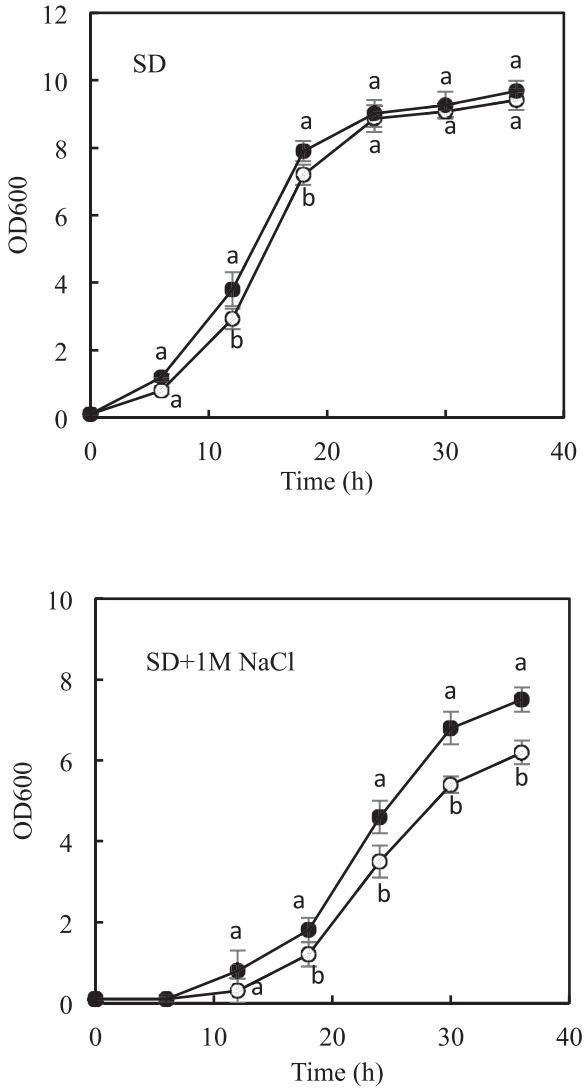

Fig. 4. Effect of GABA application on cell growth.

C. glycerinogenes UA5 was cultured in SD medium with, or without, 1 M NaCl for 16 h at 37°C. Filled circle, 5 mM GAGA application; Open circle, control. All the values are the means of three biological replicates ± standard deviation. Letters indicate significant differences ($p < 0.05$, Duncan's multiple range test).

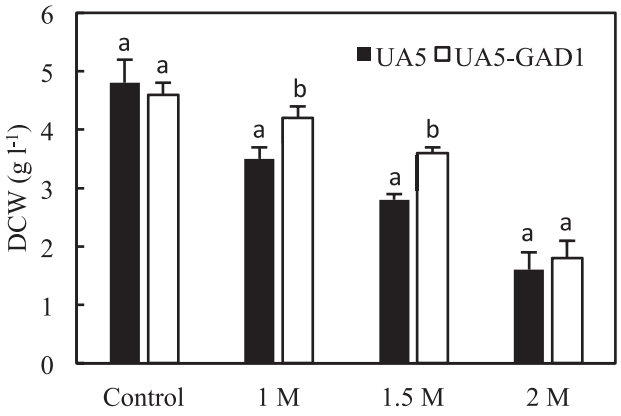

Fig. 5. Cell growth of *C. glycerinogenes* UA5 and UA5 overexpressing *GAD1* (UA5-GAD1) under hyperosmotic conditions.

Yeast cells were cultured in YPD medium supplemented with various concentrations of NaCl for 48 h at 37°C. All the values were the means of three biological replicates ± standard deviation. Letters indicate significant differences of different strains ($p < 0.05$, Duncan's multiple range test).

UGA4 was highly induced by the increasing osmolarity (Fig. 2), a finding has not been reported in other yeasts. Unlike the GABA permease genes, the expression of GABA synthesis gene *GAD1* was not induced by osmotic stress. In YPD medium, similar expression profiles were observed for GABA permease and synthesis genes (Fig. S1). Since Gap1 is not a specific GABA permease, but has multiple roles in transmembrane transport of amino acid such as the osmolytes proline, the upregulation of *GAP1* could also have implications for the uptake of proline (Matsuura and Takagi, 2005).

Extracellular addition of GABA promotes cell growth of C. glycerinogenes under hyperosmotic stress

To verify if the GABA accumulates intracellularly under hyperosmotic stress, *C. glycerinogenes* cells were cultured in the SD medium containing NaCl, with, or without, exogenous 5 mM GABA application. The intracellular GABA contents were significantly enhanced with increasing osmolarity (Fig. 3), confirming that the GABA accumulation was induced by osmotic stress, in accordance with the upregulated expression of GABA permeases genes. Cells exposed to the same cultures without GABA addition were found to contain trace amounts of intracellular GABA (Fig. 3). These results suggested that the accumulation of high GABA levels does occur in *C. glycerinogenes* under hyperosmotic conditions, when there is available GABA in the environment.

To clarify the effect of exogenous GABA application on cell growth, 5 mM GABA was added into the SD medium with, or without, 1 M NaCl (Fig. 4). In the absence of NaCl, the application of GABA had no effect on the cell growth of *C. glycerinogenes*. However, a significant improvement of cell growth was observed under hyperosmotic conditions, highlighting the role of GABA to protect *C. glycerinogenes* cells from hyperosmotic stress. A similar study in black pepper plants proved that pretreatment with 2 mM GABA has a priming effect in inducing osmotic stress tolerance, however no prior related studies were performed in yeasts (Vijayakumari and Puthur, 2015). It has also been shown that a large proportion of GABA was stored within yeast cells, indicating a possible role in stress tolerance mechanism, similar to that of arginine and proline (Bach et al., 2009; Matsuura and Takagi, 2005). Our results verifies the hypothesis that GABA application increases the intracellular GABA accumulation and improves the osmotic tolerance in *C. glycerinogenes* cells, as it does in plant cells.

Overexpression of GAD1 enhances the cell growth under hyperosmotic stress

In addition to exogenous GABA application, enhancing GABA synthesis by the overexpression of the key enzyme gene *GAD1* encoding glutamate decarboxylase is an alternative procedure to improve cell growth under hyperosmotic conditions. In the present study, the endogenous *GAD1* gene was overexpressed in *C. glycerinogenes* under a strong glyceraldehyde-3-phosphate dehydrogenase

promoter. As expected, the intracellular GABA was increased by 75.9% (Table S1), and the recombinant UA5-GAD1 showed better growth performance under hyperosmotic conditions (Fig. 5). The DCW values of UA5-GAD1 were increased by 20% (YPD supplemented with 1 M NaCl) and 28.6% (YPD supplemented with 1.5 M NaCl) compared with the wild strain UA5 under the same conditions. However, when encountered with extremely hyperosmotic conditions (2 M NaCl), there was no detectable enhancement of cell growth. These results confirm that GABA accumulation plays an important role for *C. glycerinogenes* in hyperosmotic stress tolerance. Rice plant cells overexpressing the glutamate decarboxylase gene also was reported to accumulate extremely high amounts of GABA (Akama and Takaiwa, 2007). The accumulation of GABA under hyperosmotic stress might be associated with an enhanced GABA shunt and acid metabolism in plants (Akcay et al., 2012; Li et al., 2017). In addition, exogenous GABA application could reduce the accumulation of harmful substances, increase antioxidant enzyme activity, and upregulate osmoprotectants such as proline and soluble sugar under salt stress (Wang et al., 2017).

In plant cells, high levels of GABA accumulate in response to various environmental stresses including osmotic stress. However, no similar physiological phenomenon has been reported in yeasts. Our study provides the first demonstration of the function of GABA accumulation in osmotic stress resistance of the unconventional yeast *C. glycerinogenes*. Improvement of intracellular GABA accumulation, either through exogenous application or cellular synthesis, is available for enhancing the cell growth of *C. glycerinogenes* under hyperosmotic conditions.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Nos. 31570052, 31601456).

Supplementary Materials

Supplementary figure and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References

Akama, K. and Takaiwa, F. (2007) C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in plant cells. *J. Exp. Bot*., **58**, 2699–2707.

Akcay, N., Bor, M., Karabudak, T., Ozdemir, F., and Turkan, I. (2012) Contribution of Gamma amino butyric acid (GABA) to salt stress responses of Nicotiana sylvestris CMSII mutant and wild type plants. *J. Plant. Physiol*., **169**, 452–458.

Bach, B., Meudec, E., Lepoutre, J. P., Rossignol, T., Blondin, B. et al. (2009) New insights into gamma-aminobutyric acid catabolism: Evidence for gamma-hydroxybutyric acid and polyhydroxybutyrate synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol*., **75**, 4231–4239.

Bouche, N. and Fromm, H. (2004) GABA in plants: just a metabolite? *Trends Plant Sci*., **9**, 110–115.

Cao, J., Barbosa, J. M., Singh, N. K., and Locy, R. D. (2013) GABA shunt mediates thermotolerance in *Saccharomyces cerevisiae* by reducing reactive oxygen production. *Yeast*, **30**, 129–144.

Chen, X. Z., Fang, H. Y., Rao, Z. M., Shen, W., Zhuge, B. et al. (2008) Cloning and characterization of a NAD(+)-dependent glycerol-3-phosphate dehydrogenase gene from *Candida glycerinogenes*, an industrial glycerol producer. *FEMS Yeast Res*., **8**, 725–734.

Coleman, S. T., Fang, T. K., Rovinsky, S. A., Turano, F. J., and Moye-Rowley, W. S. (2001) Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. *J. Biol. Chem*., **276**, 244–250.

Guan, N., Li, J., Shin, H. D., Du, G., Chen, J. et al. (2017) Microbial response to environmental stresses: from fundamental mechanisms to practical applications. *Appl. Microbiol. Biotechnol*., **101**, 3991–4008.

Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev*., **66**, 300–372.

Ji, H., Zhuge, B., Zong, H., Lu, X., Fang, H. et al. (2016) Role of CgHOG1 in stress responses and glycerol overproduction of *Candida glycerinogenes*. *Curr. Microbiol*., **73**, 827–833.

Ji, H., Lu, X., Zong, H., and Zhuge, B. (2017) A synthetic hybrid promoter for D-xylonate production at low pH in the tolerant yeast *Candida glycerinogenes*. *Bioengineered*, 1–7.

Kaino, T. and Takagi, H. (2008) Gene expression profiles and intracellular contents of stress protectants in *Saccharomyces cerevisiae* under ethanol and sorbitol stresses. *Appl. Microbiol. Biotechnol*., **79**, 273–283.

Karatzas, K. A., Brennan, O., Heavin, S., Morrissey, J., and O'Byrne, C. P. (2010) Intracellular accumulation of high levels of gamma-aminobutyrate by *Listeria monocytogenes* 10403S in response to low pH: uncoupling of gamma-aminobutyrate synthesis from efflux in a chemically defined medium. *Appl. Environ. Microbiol*., **76**, 3529–3537.

Kinnersley, A. M. and Turano, F. J. (2000) Gamma Aminobutyric Acid (GABA) and plant responses to stress. *Critical Reviews in Plant Sciences*, **19**, 479–509.

Li, Z., Yu, J., Peng, Y., and Huang, B. (2017) Metabolic pathways regulated by abscisic acid, salicylic acid and gamma-aminobutyric acid in association with improved drought tolerance in creeping bentgrass (*Agrostis stolonifera*). *Physiol. Plant*, **159**, 42–58.

Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$ CT method. *Methods*, **25**, 402–408.

Matsuura, K. and Takagi, H. (2005) Vacuolar functions are involved in stress-protective effect of intracellular proline in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng*., **100**, 538–544.

Parmar, J. H., Bhartiya, S., and Venkatesh, K. V. (2011) Characterization of the adaptive response and growth upon hyperosmotic shock in *Saccharomyces cerevisiae*. *Mol. Biosyst*., **7**, 1138–1148.

Remize, F., Cambon, B., Barnavon, L., and Dequin, S. (2003) Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by the HOG pathway. *Yeast*, **20**, 1243–1253.

Rep, M. (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J. Biol. Chem*., **275**, 8290–8300.

Rep, M., Albertyn, J., Thevelein, J. M., Prior, B. A., and Hohmann, S. (1999) Different signalling pathways contribute to the control of *GPD1* gene expression by osmotic stress in *Saccharomyces cerevisiae*. *Microbiology*, **145**, 715–727.

Saito, H. and Posas, F. (2012) Response to hyperosmotic stress. *Genetics*, **192**, 289–318.

Saxena, A. and Sitaraman, R. (2016) Osmoregulation in *Saccharomyces cerevisiae* via mechanisms other than the high-osmolarity glycerol pathway. *Microbiology*, **162**, 1511–1526.

Siderius, M. H., Wuytswinkel, V. O., Reijenga, K. A., Kelders, M., and Mager, W. H. (2000) The control of intracellular glycerol in *Saccharomyces cerevisiae* influences osmotic stress response and resistance to increased temperature. *Mol. Microbiol*., **36**, 1381–1390.

Takagi, H. (2008) Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. *Appl. Microbiol. Biotechnol*., **81**, 211–223.

Takagi, H., Taguchi, J., and Kaino, T. (2016) Proline accumulation protects *Saccharomyces cerevisiae* cells in stationary phase from ethanol stress by reducing reactive oxygen species levels. *Yeast*, **33**, 355–363.

Vijayakumari, K. and Puthur, J. T. (2015) γ-Aminobutyric acid (GABA) priming enhances the osmotic stress tolerance in *Piper nigrum* Linn. plants subjected to PEG-induced stress. *Plant Growth Regulation*, **78**, 57–67.

Wang, X., Li, B. Z., Ding, M. Z., Zhang, W. W., and Yuan, Y. J. (2013) Metabolomic analysis reveals key metabolites related to the rapid adaptation of *Saccharomyce cerevisiae* to multiple inhibitors of furfural, acetic acid, and phenol. *OMICS*, **17**, 150–159.

Wang, Y., Gu, W., Meng, Y., Xie, T., Li, L. et al. (2017) Gamma-aminobutyric acid imparts partial protection from salt stress injury to maize seedlings by omproving photosynthesis and upregulating osmoprotectants and antioxidants. *Sci. Rep.*, **7**, 43609.

Xu, S., Zhou, J., Liu, L., and Chen, J. (2010) Proline enhances *Torulopsis glabrata* growth during hyperosmotic stress. *Biotechnol. Bioprocess Eng.*, **15**, 285–292.

Xu, S., Zhou, J., Liu, L., and Chen, J. (2011) Arginine: A novel compatible solute to protect *Candida glabrata* against hyperosmotic stress. *Process Biochem.*, **46**, 1230–1235.

Zhuge, J., Fang, H. Y., Wang, Z. X., Chen, D. Z., Jin, H. R. et al. (2001) Glycerol production by a novel osmotolerant yeast *Candida glycerinogenes*. *App. Microbiol. Biotechnol.*, **55**, 686–692.