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Overexpression of *OLE1* Enhances Cytoplasmic Membrane Stability and Confers Resistance to Cadmium in *Saccharomyces cerevisiae*

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ABSTRACT The heavy metal cadmium is widely used and released into the environment, posing a severe threat to crops and humans. *Saccharomyces cerevisiae* is one of the most commonly used organisms in the investigation of environmental metal toxicity. We investigated cadmium stress and the adaptive mechanisms of yeast by screening a genome-wide essential gene overexpression library. A candidate gene, *OLE1*, encodes a delta-9 desaturase and was associated with high anti-cadmium-stress activity. The results demonstrated that the expression of *OLE1* was positively correlated with cadmium stress tolerance and induction was independent of Mga2p and Spt23p (important regulatory factors for *OLE1*). Moreover, in response to cadmium stress, cellular levels of monounsaturated fatty acids were increased. The addition of exogenous unsaturated fatty acids simulated overexpression of *OLE1*, leading to cadmium resistance. Such regulation of *OLE1* in the synthesis of unsaturated fatty acids may serve as a positive feedback mechanism to help cells counter the lipid peroxidation and cytoplasmic membrane damage caused by cadmium.

IMPORTANCE A *S. cerevisiae* gene encoding a delta-9 desaturase, *OLE1*, was associated with high anti-cadmium-stress activity. The data suggest that the regulation of *OLE1* in the synthesis of unsaturated fatty acids may serve as a positive feedback mechanism to help yeast cells counter the lipid peroxidation and cytoplasmic membrane damage caused by cadmium. The discovery of *OLE1* involvement in membrane stability may indicate a novel defense strategy against cadmium stress.

KEYWORDS cadmium, *OLE1*, delta-9 desaturase, unsaturated fatty acids, cytoplasmic membrane

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As a result of various industrial activities, such as mining, pouring, casting, and processing, pollution with toxic metals has become a global environmental problem. These environmental metals are taken up by rooted crops, accumulate, and later enter the human body through the food chain (1, 2). Among the metals, cadmium is one of the toxic metals. Cadmium not only inhibits crop growth and reduces crop yields but also threatens human health; it has been classified by the International Agency for Research on Cancer as a category I human carcinogen (3). Chronic exposure to cadmium may cause damage to a variety of human organs, including the cardiovascular, immune, and reproductive systems (4, 5). However, the mechanisms of environmental cadmium-induced stress and damage are unclear.

Previous investigations have demonstrated that cadmium induces cellular apoptosis by altering the balance of certain essential cations and trace elements (6, 7) and disturbing signal transduction (8). *CAD2*, which encodes a P-type cation-transporting

ATPase, could confer cadmium resistance to cells (9). Other detoxification mechanisms are also involved in cadmium resistance in cells (9–11), including lipid peroxidation (12). However, few studies on the involvement of lipid-associated pathways in cadmium damage or resistance mechanisms have been reported.

Several microorganisms, such as yeast and bacteria, have been investigated for their use in different environments for metal toxicity treatment and control (6). One of the most commonly used models in such investigations is yeast. As a model eukaryote, the yeast *Saccharomyces cerevisiae* is a very useful experimental system for obtaining an integrated assessment of and genome-wide perspective on yeast responses to environmental toxicants (13). Three types of fitness-based assays can be used to identify toxicant-induced phenotypes, including homozygous (knockout deletion; gene dosage of 0%), haploinsufficiency (heterozygous deletion; gene dosage of 50%), and multicopy and overexpression (gene dosage of >100%) screens (14). Among these assays, overexpression screening assays are powerful tools that can be used to identify genes or genetic pathways that confer resistance to a compound and to explore gene function (15, 16).

To determine the mechanisms of cadmium resistance and to control cadmium pollution, it is necessary to explore the cadmium damage and tolerance mechanisms of yeast cells. In the present study, a yeast essential gene overexpression library was screened for genes involved in the anti-cadmium-stress response. The findings provide the theoretical basis for understanding cadmium stress mechanisms and cadmium pollution control.

RESULTS

***OLE1* overexpression confers increased resistance to cadmium.** In a previous study, we observed that yeast was sensitive to cadmium; 150 μM was the approximate lethal dose (ALD) of cadmium for BY4741 cells, and cadmium exhibited strong inhibitory effects on the growth of yeast (10). In this study, 300 μM cadmium (2 times the ALD) was used to treat yeast harboring the essential gene overexpression library, in order to screen for anti-cadmium-stress genes. Ten strains survived on synthetic complete minus uracil (SC−Ura) plates with 300 μM cadmium and were isolated; the plasmid DNAs were recovered from these strains (Fig. 1A). Three plasmids harboring cadmium resistance genes, i.e., *OLE1*, *PRP16*, and *PCL8*, were identified by DNA sequencing. Among these plasmid-bearing strains, the one carrying *OLE1* exhibited the highest levels of resistance to cadmium (Fig. 1B). To exclude cadmium resistance induced by secondary mutations during the screening process, the plasmid carrying *OLE1* was transformed into the original wild-type strains BY4741 and BY4742; the strains bearing the *OLE1* plasmid both showed strong cadmium resistance (Fig. 1C).

Several cadmium resistance genes are involved in the efflux of cadmium to affect cadmium homeostasis, resulting in the reduction of intracellular cadmium accumulation (9, 17). To identify the cadmium resistance of *OLE1*, we measured the effect of *OLE1* overexpression on intracellular levels of cadmium in yeast by using inductively coupled plasma mass spectrometry (ICP-MS). That assessment showed that cadmium exposure resulted in a significant increase in the intracellular cadmium level, compared to the control. However, overexpression of *OLE1* did not reduce the intracellular cadmium levels of yeast (Fig. 1D). The results suggest that *OLE1* is not involved in the efflux of cadmium.

OLE1 has been reported to code for a desaturase that catalyzes the desaturation of delta-9 fatty acids ($C_{16:0}$ and $C_{18:0}$) (18) and produces the endogenous monounsaturated fatty acids, such as stearic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) (18, 19). Thus, we hypothesized that *OLE1* is involved in cadmium stress tolerance in yeast.

***OLE1* was upregulated in response to cadmium stress.** In support of the aforementioned hypothesis, the correlation between *OLE1* expression levels and cadmium resistance was investigated. Because the *OLE1* null mutant is nonviable under standard conditions and a deficiency of *MGA2* or *SPT23* (important transcriptional regulators of *OLE1*) results in serious reduction of *OLE1* expression levels (20), cadmium resistance

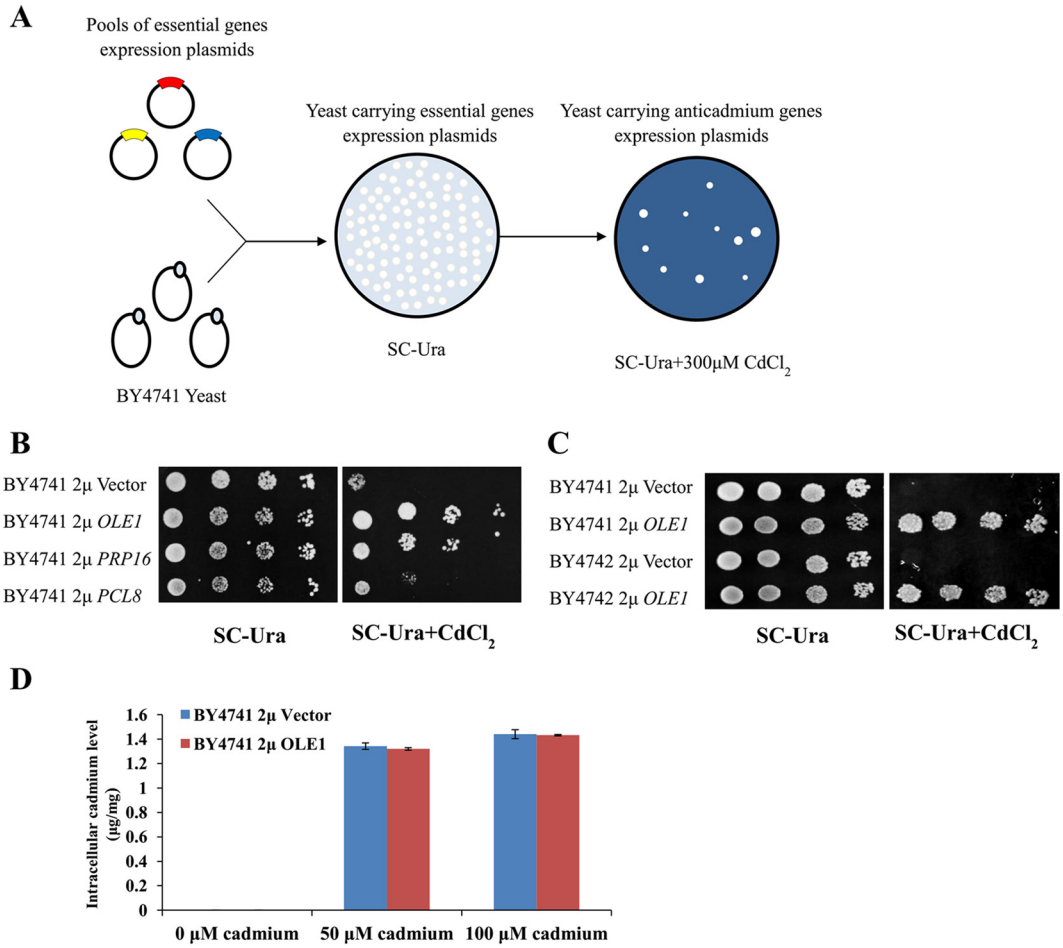

FIG 1 Screen for anti-cadmium-stress genes and confirmation tests. (A) Schematic of genetic screening with the yeast essential gene library. Different colors in the plasmids represent different yeast essential genes. The anti-cadmium-stress gene screen was performed as described in Materials and Methods. (B and C) Growth of yeast strains with and without cadmium. (B) Strains carrying anti-cadmium-stress genes were grown for 2 to 5 days on SC−Ura plates with or without the addition of 300 μM $CdCl_2$, 10-fold serially diluted, and then spotted on plates for confirmation of cadmium resistance. (C) Wild-type strains (BY4741 and BY4742) transformed with the *OLE1* plasmid were grown for 2 to 5 days on SC−Ura plates with or without the addition of 300 μM $CdCl_2$, to ensure that there were no secondary mutations that induced cadmium resistance in yeast and to confirm that *OLE1* could induce cadmium resistance. (D) Effect of *OLE1* on intracellular cadmium levels. BY4741 carrying the vector (blue) or the OLE1 (red) plasmid was tested after treatment with 0, 50, or 100 μM $CdCl_2$ for 12 h, as described in Materials and Methods. Values are means and standard deviations ($n = 3$).

tests were carried out with *mga2*Δ and *spt23*Δ mutants. As shown in Fig. 2A, the strain with the deletion of *MGA2* showed high sensitivity to 100 μM cadmium and the strain with the deletion of *SPT23* showed low sensitivity to 100 μM cadmium, compared to the *mga2*Δ strain. However, the cadmium resistance of both the *mga2*Δ and *spt23*Δ strains was recovered after overexpression of *OLE1* (Fig. 2B), which suggests that there was a positive correlation between the *OLE1* expression level and cadmium resistance.

Using reverse transcription (RT)-PCR, the relative *OLE1* mRNA levels in wild-type, *mga2*Δ, and *spt23*Δ strains were quantified. The data are in accordance with those in the previous report (21). That is, the *OLE1* mRNA levels in the *mga2*Δ and *spt23*Δ strains were lower than the level in the wild-type strain (Fig. 2C and D). Thus, strains with low *OLE1* expression levels exhibit high sensitivity to cadmium. In the presence of 50 μM cadmium for 3 h, the *OLE1* mRNA levels were significantly increased in the wild-type, *mga2*Δ, and *spt23*Δ strains (Fig. 2B and C). This suggests that cadmium induces the upregulation of *OLE1* independently of *MGA2* and *SPT23*.

Increased monounsaturated fatty acid contents were induced in response to cadmium stress. According to previous studies, almost all of the endogenous mono-

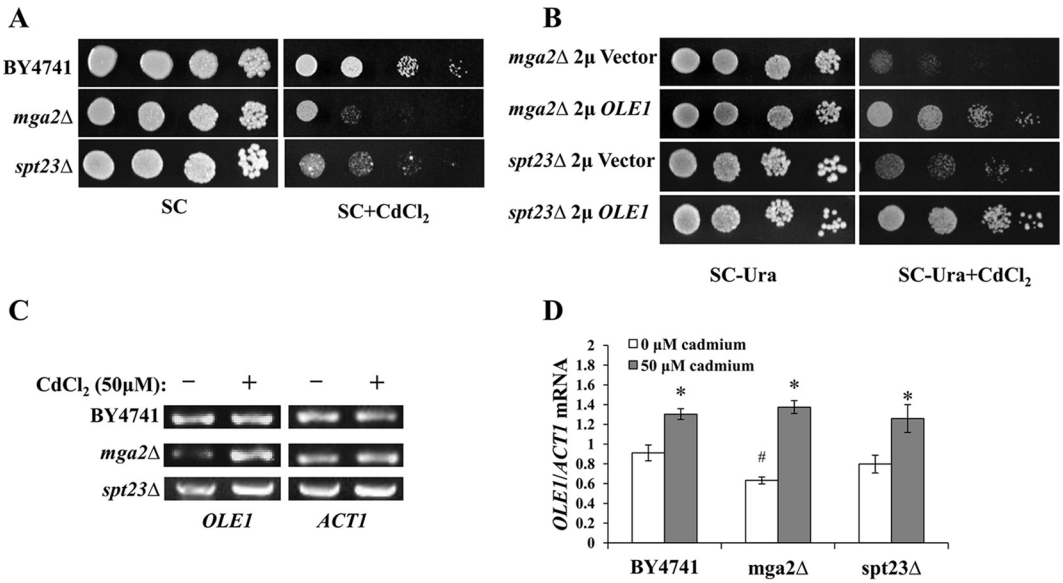

FIG 2 *OLE1* expression regulation and cadmium stress resistance. (A and B) Growth of yeast strains with and without cadmium. Yeast cultures were 10-fold serially diluted and then spotted on plates. (A) BY4741, *mga2*Δ, and *spt23*Δ cells were grown on SC−Ura plates with or without the addition of 100 μM $CdCl_2$ and were incubated at 30°C for 2 to 5 days for the cadmium stress resistance test. (B) *mga2*Δ and *spt23*Δ cells carrying vector or the *OLE1* plasmid were grown on SC−Ura plates with or without the addition of 100 μM $CdCl_2$ and were incubated at 30°C for 2 to 5 days for confirmation of the positive correlation between *OLE1* expression levels and cadmium resistance. (C) RT-PCR analysis of *OLE1* mRNA levels in BY4741, *mga2*Δ, and *spt23*Δ yeast before and after exposure to 50 μM $CdCl_2$. (D) Quantification of the semiquantitative RT-PCR analysis in panel C. Values are means and standard deviations ($n = 3$). *, significantly different from the corresponding controls ($P < 0.05$); #, significantly different from the BY4741 control that was not treated with $CdCl_2$ ($P < 0.05$).

unsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) are produced from saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) by the delta-9 desaturase encoded by *OLE1* (Ole1p) (19, 22, 23). To explore the involvement of *OLE1* in cadmium stress, the intracellular contents of saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) and monounsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) were analyzed using gas chromatography-mass spectrometry (GC-MS). As shown in Fig. 3A, the overexpression of *OLE1* in wild-type yeast resulted in a 35.4% increase in the content of monounsaturated fatty acids and a 47.4% decrease in the content of saturated fatty acids, indicating that a high level of *OLE1* contributes to the conversion of saturated fatty acids to monounsaturated fatty acids. As expected, exposure to cadmium in wild-type yeast also caused increases in the monounsaturated fatty acid content and decreases in the saturated fatty acid content, in a concentration-dependent manner (Fig. 3B). In the *OLE1* overexpression strain, however, cadmium did not induce any significant change in the contents of saturated and monounsaturated fatty acids (Fig. 3C). Furthermore, using oleic acid ($C_{18:1}$) as an example, as shown in Fig. 3D, both overexpression of *OLE1* and exposure to cadmium in wild-type yeast resulted in significant increases in the oleic acid content. Similar to the findings in Fig. 3C, cadmium did not induce any significant change in the content of oleic acid in the *OLE1* overexpression strain (Fig. 3D). Because the strain carrying *OLE1* exhibited high levels of resistance to cadmium (Fig. 1B and C), the increase in the content of monounsaturated fatty acids may alleviate cadmium-induced stress.

Oleic acid alleviated cadmium-induced membrane damage in yeast. Oleic acid ($C_{18:1}$), one of the main products of the desaturase Ole1p, is an important component of cell membranes (24) and is related to membrane fluidity (4). Cadmium was reported to induce extensive membrane damage in plant cells by altering membrane fluidity (21, 25, 26). To explore the relationship between monounsaturated fatty acids and cadmium stress, the effects of oleic acid on membrane integrity in different yeast strains treated with cadmium were investigated. Here, propidium iodide (PI) was used as a fluorescent dye in the membrane integrity analysis (27). As shown in Fig. 4A and B, the percentage of dead cells (PI positive) treated with cadmium was over 30%, compared to the control.

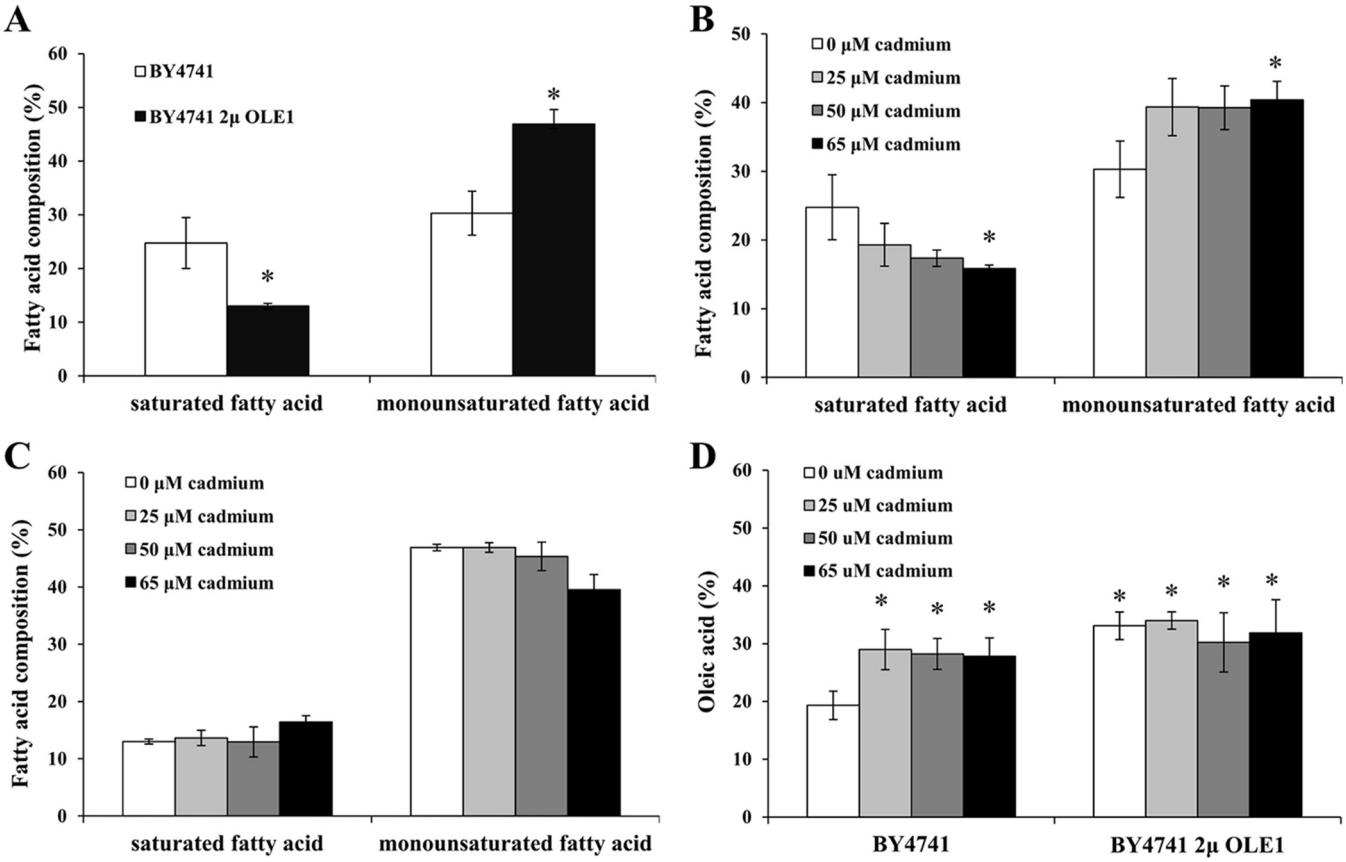

FIG 3 Effects of cadmium on saturated and monounsaturated fatty acid contents. (A) The saturated and monounsaturated fatty acid contents were analyzed in the yeast BY4741 and in the strain with *OLE1* overexpression, as described in Materials and Methods. (B and C) The fatty acid contents in yeast BY4741 (B) and the strain with overexpression of *OLE1* (C) were analyzed after treatment for 18 h with 0, 25, 50, or 65 μM $CdCl_2$. (D) The oleic acid contents in yeast BY4741 and the strain with *OLE1* overexpression were analyzed after treatment for 18 h with 0, 25, 50, or 65 μM $CdCl_2$. Values are means and standard deviations ($n = 3$). *, significantly different from the BY4741 control that was not treated with $CdCl_2$ ($P < 0.05$).

Percentages were significantly decreased after the addition of oleic acid or the overexpression of *OLE1*, to 19.6% or 18.3%, respectively. To confirm the relationship between monounsaturated fatty acids and cadmium stress, a similar investigation was performed with the *mga2*Δ strain. As expected, cadmium induced a high percentage of dead cells, nearly 60% higher than that in the *mga2*Δ control. The addition of oleic acid or the overexpression of *OLE1* could significantly decrease the PI percentage. At the same time, compared to wild-type yeast BY4741, the *mga2*Δ strain showed greater sensitivity to cadmium.

To confirm the results, we analyzed the effects of oleic acid on cadmium stress by spot testing the wild-type and *mga2*Δ strains. After the addition of oleic acid, both strains exhibited high anti-cadmium-stress capacity (Fig. 4C and 5). Taken together, these data clearly demonstrate that oleic acid alleviates cadmium-induced membrane damage in yeast.

***OLE1* reduced lipid peroxidation.** Previous studies showed that cadmium toxicity was associated with lipid peroxidation (12). Lipid peroxidation can trigger cell membrane damage; therefore, the prevention of cadmium-induced lipid peroxidation was a useful strategy for attenuating cadmium toxicity (12). Because our data showed that the lipid desaturase-associated gene *OLE1* and oleic acid could prevent membrane damage caused by cadmium in yeast, we hypothesized that *OLE1* might reduce the level of cadmium-induced lipid peroxidation. To gain deeper insight into the cadmium resistance mechanisms of *OLE1* and oleic acid, the effects of *OLE1* and oleic acid on cadmium-induced lipid peroxidation were tested by measuring the levels of thiobarbituric acid reactive substances (TBARS), a commonly used indicator for evaluation of

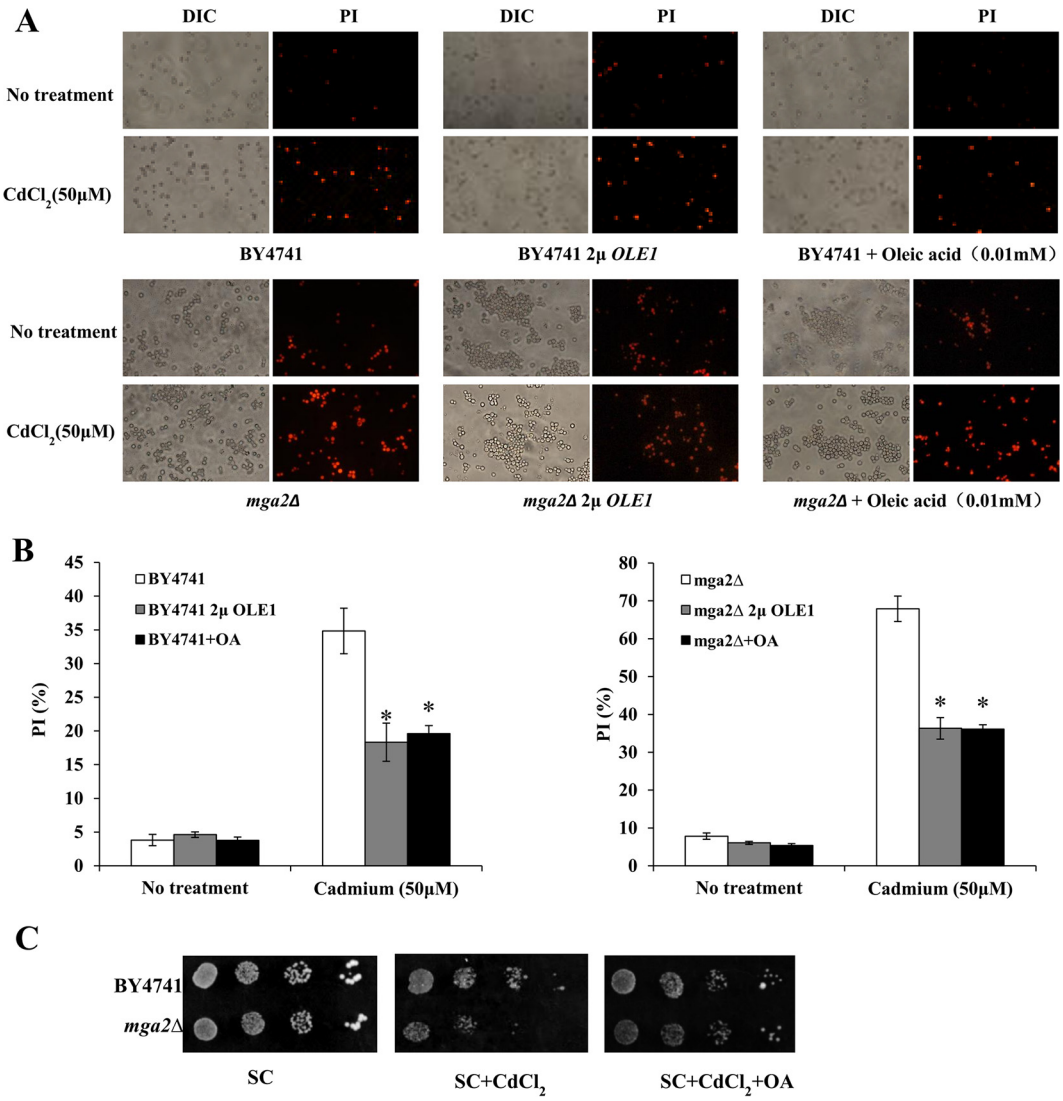

FIG 4 Protective effects of oleic acid on membrane integrity and cadmium stress resistance. (A) Representative photographs of cells treated for 3 h with or without cadmium. The indicated cells were incubated for 3 h with 0 or 50 μM $CdCl_2$ or 50 μM $CdCl_2$ with 0.01 mM oleic acid and then were stained and visualized as described in Materials and Methods. DIC, differential interference contrast. (B) Quantification of the percentages of PI-stained cells shown in panel A. Values are means and standard errors of the means ($n = 3$). *, significantly different from the controls (BY4741 or *mga2*Δ cells treated with $CdCl_2$) ($P < 0.05$). OA, oleic acid. (C) Growth of yeast cells with and without cadmium. Cultures of BY4741 and *mga2*Δ cells were 10-fold serially diluted and then spotted on SC−Ura plates containing 0 or 150 μM $CdCl_2$ or 150 μM $CdCl_2$ with 0.01 mM oleic acid, which were incubated at 30°C for 2 to 5 days to test for cadmium stress resistance.

lipid peroxidation. As shown in Fig. 5A and B, the concentrations of TBARS were significantly increased after treatment with cadmium in both the wild-type yeast BY4741 and the *mga2*Δ strain, and there were obvious decreases following the overexpression of *OLE1* or the addition of oleic acid. These data prove that *OLE1* and oleic acid can alleviate cadmium-induced membrane damage through the inhibition of lipid peroxidation induced by cadmium.

DISCUSSION

Several previous investigations reported that cadmium can exert toxic effects through inhibition of ATPase activity (28), interference with cation uptake (10), and induction of cellular apoptosis (29–31). Many cation uptake and transport genes were found to play important roles in cadmium resistance in plants or microorganisms (9). Shimo et al. (32) found that knockout of the *LCD* gene could reduce the concentration of cadmium and confer resistance to cadmium in rice. Additionally, impairment of lipid

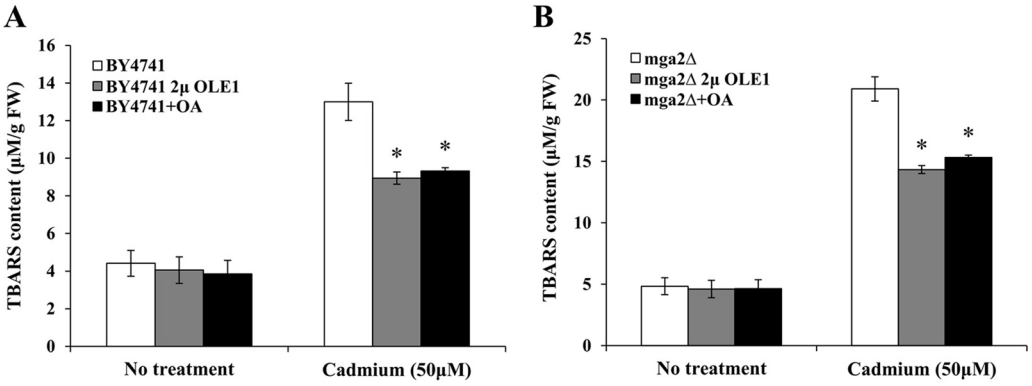

FIG 5 Effects of oleic acid on lipid peroxidation. BY4741 (A) and *mga2*Δ (B) yeast cells with or without OLE1 overexpression were incubated for 12 h with 0 or 50 μM $CdCl_2$ or 50 μM $CdCl_2$ with 0.01 mM oleic acid (OA), and then TBARS contents were measured as described in Materials and Methods. Values are means and standard deviations ($n = 3$). FW, fresh weight; *, significantly different from the controls (BY4741 or *mga2*Δ cells treated with $CdCl_2$) ($P < 0.05$).

storage and membrane damage is an important aspect of cadmium stress (33). Nouairi et al. (34) found a change in lipid composition was also an efficient defense strategy against cadmium stress in *Brassica juncea* and *Brassica napus*. Similarly, changes in lipid composition and membrane fluidity have been demonstrated in plants subjected to cadmium stress (35). Therefore, some lipid-associated pathways may be involved in the mechanisms of cadmium damage and resistance.

The overexpression screening assay in yeast provides a useful tool to identify the responses of genes or genetic pathways to environmental toxicants (13, 15, 16). In our study, we obtained 10 cadmium-tolerant colonies carrying plasmids containing three different genes, i.e., *OLE1*, *PRP16*, and *PCL8* (Fig. 1). *PRP16* encodes an RNA helicase that is involved in pre-mRNA processing (36), and *PCL8* encodes a cyclin that interacts with Pho85p cyclin-dependent kinase (Cdk) to phosphorylate and to regulate glycogen synthase (37). According to several previous reports, these two genes probably contribute to alleviating both cadmium-induced DNA/RNA damage (3) and glycogen metabolism impairment (38). Our results demonstrate that the lipid desaturase-associated gene *OLE1* is involved in cadmium stress tolerance. Compared with wild-type BY4741 (gene dosage of 100%), overexpression of *OLE1* (gene dosage of >100%) conferred high-level cadmium stress tolerance and low levels of expression of *OLE1* (*mga2*Δ; gene dosage of <100%) yielded sensitivity to cadmium stress (Fig. 1B and 2). On the other hand, *OLE1* was upregulated in response to cadmium stress (Fig. 2C and D). Therefore, *OLE1* expression levels were positively correlated with cadmium resistance in yeast. However, a study of the mechanisms of *OLE1* involvement in cadmium resistance has not been reported.

Controlling intracellular cadmium levels to confer resistance to cadmium is a possible function of cadmium resistance genes, such as *CAD2* (9). In this study, however, overexpression of *OLE1* did not reduce the intracellular cadmium levels of yeast after treatment with cadmium, compared to the control, suggesting that yeast cadmium resistance involving *OLE1* is irrelevant to the efflux of cadmium.

Ole1p (delta-9 fatty acid desaturase), encoded by *OLE1*, plays a role in the desaturation of saturated fatty acids (18). Almost all the endogenous monounsaturated fatty acids ($C_{16:1}$ and $C_{18:1}$) are produced from saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) by Ole1p (19, 22, 23). Our results confirmed this point. The overexpression of *OLE1* in wild-type yeast led to an increase in the content of monounsaturated fatty acids and a decrease in the content of saturated fatty acids (Fig. 3A). We also compared the changes in the contents of the two types of fatty acids in the wild-type and *OLE1* overexpression strains after cadmium treatment. Our data showed that cadmium caused an increase in the content of monounsaturated fatty acids in the wild-type strain, while it did not induce any significant change in the fatty acid contents in the *OLE1* overexpression

strain (Fig. 3C and D). Thus, *OLE1* is involved in cadmium stress tolerance through the regulation of fatty acid composition.

In general, changes in membrane fluidity and integrity resulted from alterations in lipid composition (4, 39, 40), especially unsaturated fatty acids (4). Unsaturated fatty acid levels were associated with membrane fluidity remodeling in stress-acclimating plants in response to abiotic and biotic stress (41). The regulation and expression of desaturase genes play important roles in the alteration of membrane lipid composition, which is an important adaptive response in plants to cope with cadmium stress (42). In our study, *OLE1* was found to be upregulated in response to cadmium stress (Fig. 2C). This suggests that the stress response regulation of *OLE1* may be an important adaptive response of yeast to counter cadmium stress.

A previous study showed that cadmium induced lipid peroxidation by enhancing lipoxygenase activity, which is involved in catalyzing lipid peroxidation by using membrane lipid components, especially unsaturated fatty acids, as substrates (43). Compared to saturated fatty acids, unsaturated fatty acids containing rich double bonds are vulnerable to damage from cadmium-induced free radicals, which cause unsaturated fatty acid peroxidation (44). The peroxidation of unsaturated fatty acids in the membrane causes damage to the cell membrane (45). From our results, overexpression of *OLE1* and the unsaturated fatty acid oleic acid could reduce the level of lipid peroxidation induced by cadmium and alleviate cadmium-induced membrane damage (Fig. 4 and 5). These findings demonstrate that overexpression of *OLE1* confers cadmium resistance to yeast by alleviating cadmium-induced lipid peroxidation by yielding more unsaturated fatty acids, which may play antioxidant roles (44).

Changes in lipid composition also represent an efficient defense strategy against cadmium stress in plants (34). The regulation and expression of desaturase genes play important roles in the alteration of membrane lipid composition, which was an important adaptive response of plants to cope with cadmium stress (42). In this study, *OLE1* was found to be upregulated in response to cadmium stress (Fig. 2C). This stress response regulation of *OLE1* may help yeast cells counter the lipid peroxidation and cytomembrane damage caused by cadmium. Mga2p and Spt23p are important transcription factors for *OLE1*. When the *mag2*Δ and *spt23*Δ strains were exposed to cadmium, however, *OLE1* could still be upregulated. *OLE1*, an oxygen-sensing gene, could be upregulated in response to oxidative stress (46). This finding suggested that regulation of *OLE1* in response to cadmium stress was associated with oxidative stress and independent of Mga2p and Spt23p.

Genes similar to *OLE1* exist in plants and animals, including the *OLE16* gene of *Zea mays* (47) and the *SCD1* gene of animals (48). In the rat liver, delta-9 desaturase encoded by *SCD1* is a target of cadmium and is significant inhibited by cadmium (49). In this case, the upregulation of *OLE1* could contribute to maintaining delta-9 desaturase levels in response to the suppression of desaturase by cadmium. Thus, our results indicate that *OLE1* confers resistance to cadmium in yeast.

In conclusion, the data we have obtained support the idea that the regulation of *OLE1* in the synthesis of unsaturated fatty acids may serve as a positive feedback mechanism to help yeast cells counter the lipid peroxidation and cytoplasmic membrane damage caused by cadmium. The discovery of *OLE1* involvement in membrane stability may indicate a novel defense strategy against cadmium stress.

MATERIALS AND METHODS

Strains and growth conditions. The strains of *S. cerevisiae* used included the wild-type strain BY4741 (*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*), BY4742 (*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*), *mga2*Δ (*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 MGA2Δ::KanMX4*), and *spt23*Δ (*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 SPT23Δ::KanMX4*). Cells were maintained on yeast peptone dextrose (YPD) medium (2.0% glucose, 2.0% peptone, 1.0% yeast extract, and 2.0% agar [wt/vol]), with or without G418, before experimental procedures. For plasmid-bearing studies, cells were grown at 30°C in synthetic complete (SC) medium (0.67% yeast nitrogen base [YNB], 2.0% glucose, and complete amino acid mixture) or SC−Ura medium. Yeast extract, peptone, glucose, $CdCl_2$, and other chemicals were purchased from Sangon Biotech (Shanghai, China) unless specifically noted otherwise.

Screening for anti-cadmium-stress genes. The *S. cerevisiae* essential gene overexpression library, which was constructed using the 2μ high-copy-number yeast-bacterium shuttle vector pXP684 in a previous study (50), was used to screen for anti-cadmium-stress genes. The library contains 1,096 genes, covering 94.8% of the 1,156 essential genes, based on the annotated *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). Briefly, the essential gene overexpression library was transformed into BY4741, and yeast cells carrying the plasmids were plated on SC−Ura plates with a high concentration (300 μM) of $CdCl_2$. The plates were incubated at 30°C for 2 to 5 days, and the cadmium-tolerant yeast colonies were isolated.

Intracellular cadmium analysis. Cells were exposed to 0, 50, or 100 μM $CdCl_2$ for 12 h at 30°C, with constant shaking, and then were harvested and washed with deionized water. Equivalent cell amounts were weighed and dried at 70°C overnight. After the dried cell weight was recorded, the samples were wet ashed in 2.0 ml HNO_3 for 1 h, in sealed vials, with heating (100°C). The digests were diluted appropriately with deionized water and analyzed for cadmium using ICP-MS (Agilent 7500CX; Agilent Technologies, Santa Clara, CA, USA). The ICP-MS analytical characteristics were as described previously (51).

Yeast cadmium sensitivity assay using the drop test. The cadmium sensitivity of yeast strains was determined using the drop test technique. Briefly, yeast cells (2×10^6) were obtained from a single clone on medium. Yeast strains were serially diluted 10-fold, spotted on SC−Ura plates supplemented with 100 or 150 μM $CdCl_2$ and/or 0.01 mM oleic acid, and incubated at 30°C for 2 to 5 days.

Saturated and monounsaturated fatty acid analysis. Fatty acids were extracted as described previously (52). Fatty acid methyl esters were prepared as described previously, with little modification (53). Fatty acids were saponified with 0.4 M KOH in methanol and mixed with 1 ml benzene-petroleum ether (1:1 [vol/vol]), followed immediately by shaking and standing. After 16 min of standing, the samples were washed with 1.6 ml of sterile water; the water was removed after stratification. The rest of the fatty acids were dried under nitrogen gas, dissolved in 200 μl of hexane, and analyzed using an Agilent 6890N gas chromatograph equipped with a 5975 mass spectrometer (Agilent). The injector temperature was 240°C. The flow rate of helium gas through the column was 1 ml/min. The column temperature was held at 80°C for 5 min and then increased to 320°C at a rate of 20°C/min. $C_{14:0}$ fatty acids (Sigma, St. Louis, MO, USA) were methyl esterified as standards prior to use. The identities of all peaks in the chromatograms were determined by comparing their retention times with those of standard fatty acid methyl esters. The fatty acid compositions were expressed as percentages of the sum of the peak areas.

RT-PCR analysis of mRNA expression. mRNA expression analysis for each gene was performed as described previously (54). Briefly, total RNA was isolated using a Yeast RNAiso kit (TaKaRa, Tokyo, Japan). One microgram of total RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). One microliter of cDNA was used as the template. *OLE1* and *ACT1* were amplified by PCR using the primer pairs OLE1-F (5′-GGCTAGAGCTGATATTACCG-3′)/OLE1-R (5′-GCGTTTCTGTAATCAGTTGG-3′) and ACT1-F (5′-GCTTTGTTCCATCCTTCTG-3′)/ACT1-R (5′-GAAACACTTGTGGTGAACG-3′), respectively. PCR products were analyzed by electrophoresis in 2% agarose gels and were quantified using the Tanon 3500 gel imaging system (Tanon Science & Technology, Shanghai, China).

Propidium iodide assay of cell membrane integrity. The cellular membrane integrity was examined by using PI, as described previously (55). Briefly, exponentially growing cells were grown in 3 ml of medium supplemented with 50 μM $CdCl_2$ and/or 0.01 mM oleic acid. After 3 h of treatment, the cells were harvested by centrifugation at 4,000 rpm for 10 min at 4°C and were washed twice with distilled water. PI (1 mg/ml) was added to 1 ml of the cell suspensions, to a final concentration of l μg/ml. After 15 min of incubation at 4°C, cells were examined by fluorescence microscopy (Olympus BX53; Olympus, Tokyo, Japan). Cells were counted, and the percentages of cells exhibiting red fluorescence were plotted. Each value was obtained from three independent experiments.

Evaluation of lipid peroxidation. Measurement of the products of lipid peroxidation was performed as described previously (56). Cells were treated for 12 h with 50 μM $CdCl_2$ and/or 0.01 mM oleic acid, harvested, and added to 1 ml of thiobarbituric acid (TBA) reagent (0.25 M HCl, 15% trichloroacetic acid, and 0.375% TBA). Addition of the reagent terminated lipid peroxidation and initiated the assay. Cells were incubated at 100°C for 15 min and then centrifuged. The absorbance of the supernatants was measured at 532 nm using a UV spectrophotometer; an equal volume of TBA reagent with distilled water was used as a blank. The levels of lipid peroxides were expressed as moles of TBARS per gram (fresh weight) of yeast.

Statistical analyses. All experiments were performed at least three times. Values were expressed as means ± standard deviations. Statistical analyses were performed using Student's *t* test. *P* values of <0.05 were considered statistically significant.

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REFERENCES

1. Apostoli P. 2002. Elements in environmental and occupational medicine. J Chromatogr B Analyt Technol Biomed Life Sci 778:63–97. https://doi.org/10.1016/S0378-4347(01)00442-X.
2. Kumar R, Chawla J, Kaur I. 2015. Removal of cadmium ion from wastewater by carbon-based nanosorbents: a review. J Water Health 13:18–33. https://doi.org/10.2166/wh.2014.024.
3. Waisberg M, Joseph P, Hale B, Beyersmann D. 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192:95–117. https://doi.org/10.1016/S0300-483X(03)00305-6.
4. Stubbs CD, Smith AD. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 779:89–137. https://doi.org/10.1016/0304-4157(84)90005-4.
5. Krivosheev AB, Poteriaeva EL, Krivosheev BN, Kupriianova L, Smirnova EL. 2012. Toxic effects of cadmium on the human body. Med Tr Prom Ekol (6):35–42. (In Russian.)
6. Mielniczki-Pereira AA, Hahn AB, Bonatto D, Riger CJ, Eleutherio EC, Henriques JA. 2011. New insights into the Ca^{2+}-ATPases that contribute to cadmium tolerance in yeast. Toxicol Lett 207:104–111. https://doi.org/10.1016/j.toxlet.2011.08.023.
7. Gomes DS, Fragoso LC, Riger CJ, Panek AD, Eleutherio EC. 2002. Regulation of cadmium uptake by Saccharomyces cerevisiae. Biochim Biophys Acta 1573:21–25. https://doi.org/10.1016/S0304-4165(02)00324-0.
8. Chen S, Xu Y, Xu B, Guo M, Zhang Z, Liu L, Ma H, Chen Z, Luo Y, Huang S, Chen L. 2011. CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death. J Neurochem 119:1108–1118. https://doi.org/10.1111/j.1471-4159.2011.07493.x.
9. Shiraishi E, Inouhe M, Joho M, Tohoyama H. 2000. The cadmium-resistant gene, *CAD2*, which is a mutated putative copper-transporter gene (*PCA1*), controls the intracellular cadmium-level in the yeast S. cerevisiae. Curr Genet 37:79–86. https://doi.org/10.1007/s002940050013.
10. Kuang X, Fang Z, Wang S, Shi P, Huang Z. 2015. Effects of cadmium on intracellular cation homoeostasis in the yeast Saccharomyces cerevisiae. Toxicol Environ Chem 97:922–930. https://doi.org/10.1080/02772248.2015.1074689.
11. Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA. 1997. A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci U S A 94:42–47. https://doi.org/10.1073/pnas.94.1.42.
12. Yiin SJ, Chern CL, Sheu JY, Lin TH. 1999. Cadmium induced lipid peroxidation in rat testes and protection by selenium. Biometals 12:353–359. https://doi.org/10.1023/A:1009277121164.
13. Whitacre JM. 2012. Biological robustness: paradigms, mechanisms, and systems principles. Front Genet 3:67. https://doi.org/10.3389/fgene.2012.00067.
14. Hoon S, St Onge RP, Giaever G, Nislow C. 2008. Yeast chemical genomics and drug discovery: an update. Trends Pharmacol Sci 29:499–504. https://doi.org/10.1016/j.tips.2008.07.006.
15. Jones GM, Stalker J, Humphray S, West A, Cox T, Rogers J, Dunham I, Prelich G. 2008. A systematic library for comprehensive overexpression screens in Saccharomyces cerevisiae. Nat Methods 5:239–241. https://doi.org/10.1038/nmeth.1181.
16. Butcher RA, Bhullar BS, Perlstein EO, Marsischky G, LaBaer J, Schreiber SL. 2006. Microarray-based method for monitoring yeast overexpression strains reveals small-molecule targets in TOR pathway. Nat Chem Biol 2:103–109. https://doi.org/10.1038/nchembio762.
17. Fuzhao N, Leifeng Z. 2015. Molecular characterization and expression pattern of a novel cadmium resistance gene of tobacco. Biosci J 31:1024–1029. https://doi.org/10.14393/BJ-v31n4a2015-26138.
18. Stukey JE, McDonough VM, Martin CE. 1990. The *OLE1* gene of Saccharomyces cerevisiae encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem 265:20144–20149.
19. Martin CE, Oh CS, Jiang Y. 2007. Regulation of long chain unsaturated fatty acid synthesis in yeast. Biochim Biophys Acta 1771:271–285. https://doi.org/10.1016/j.bbalip.2006.06.010.
20. Chellappa R, Kandasamy P, Oh CS, Jiang Y, Vemula M, Martin CE. 2001. The membrane proteins, Spt23p and Mga2p, play distinct roles in the activation of Saccharomyces cerevisiae *OLE1* gene expression: fatty acid-mediated regulation of Mga2p activity is independent of its proteolytic processing into a soluble transcription activator. J Biol Chem 276:43548–43556. https://doi.org/10.1074/jbc.M107845200.
21. Shah K, Singh P, Nahakpam S. 2013. Effect of cadmium uptake and heat stress on root ultrastructure, membrane damage and antioxidative response in rice seedlings. J Plant Biochem Biotechnol 22:103–112. https://doi.org/10.1007/s13562-012-0116-3.
22. Wilson RA, Chang PK, Dobrzyn A, Ntambi JM, Zarnowski R, Keller NP. 2004. Two Δ9-stearic acid desaturases are required for Aspergillus nidulans growth and development. Fungal Genet Biol 41:501–509. https://doi.org/10.1016/j.fgb.2003.12.009.
23. Nakamura MT, Nara TY. 2004. Structure, function, and dietary regulation of Δ6, Δ5, and Δ9 desaturases. Annu Rev Nutr 24:345–376. https://doi.org/10.1146/annurev.nutr.24.121803.063211.
24. Stukey JE, McDonough VM, Martin CE. 1989. Isolation and characterization of *OLE1*, a gene affecting fatty acid desaturation from Saccharomyces cerevisiae. J Biol Chem 264:16537–16544.
25. Rahoui S, Chaoui A, El Ferjani E. 2010. Membrane damage and solute leakage from germinating pea seed under cadmium stress. J Hazard Mater 178:1128–1131. https://doi.org/10.1016/j.jhazmat.2010.01.115.
26. Fodor E, Szabó-Nagy A, Erdei L. 1995. The effects of cadmium on the fluidity and H^+-ATPase activity of plasma membrane from sunflower and wheat roots. J Plant Physiol 147:87–92. https://doi.org/10.1016/S0176-1617(11)81418-5.
27. Moore A, Donahue CJ, Bauer KD, Mather JP. 1998. Simultaneous measurement of cell cycle and apoptotic cell death. Methods Cell Biol 57:265–278. https://doi.org/10.1016/S0091-679X(08)61584-8.
28. Astolfi S, Zuchi S, Passera C. 2005. Effect of cadmium on H^+ATPase activity of plasma membrane vesicles isolated from roots of different S-supplied maize (*Zea mays* L.) plants. Plant Sci 169:361–368. https://doi.org/10.1016/j.plantsci.2005.03.025.
29. Nargund AM, Avery SV, Houghton JE. 2008. Cadmium induces a heterogeneous and caspase-dependent apoptotic response in Saccharomyces cerevisiae. Apoptosis 13:811–821. https://doi.org/10.1007/s10495-008-0215-8.
30. Habeebu SS, Liu J, Klaassen CD. 1998. Cadmium-induced apoptosis in mouse liver. Toxicol Appl Pharmacol 149:203–209. https://doi.org/10.1006/taap.1997.8334.
31. el Azzouzi B, Tsangaris GT, Pellegrini O, Manuel Y, Benveniste J, Thomas Y. 1994. Cadmium induces apoptosis in a human T cell line. Toxicology 88:127–139. https://doi.org/10.1016/0300-483X(94)90115-5.
32. Shimo H, Ishimaru Y, An G, Yamakawa T, Nakanishi H, Nishizawa NK. 2011. Low cadmium (*LCD*), a novel gene related to cadmium tolerance and accumulation in rice. J Exp Bot 62:5727–5734. https://doi.org/10.1093/jxb/err300.
33. Pierron F, Baudrimont M, Bossy A, Bourdineaud JP, Brethes D, Elie P, Massabuau JC. 2007. Impairment of lipid storage by cadmium in the European eel (Anguilla anguilla). Aquat Toxicol 81:304–311. https://doi.org/10.1016/j.aquatox.2006.12.014.
34. Nouairi I, Ammar WB, Youssef NB, Daoud DBM, Ghorbal MH, Zarrouk M. 2006. Comparative study of cadmium effects on membrane lipid composition of Brassica juncea and Brassica napus leaves. Plant Sci 170:511–519. https://doi.org/10.1016/j.plantsci.2005.10.003.
35. Devi SR, Prasad MNV. 2004. Membrane lipid alterations in heavy metal exposed plants, p 127–145. *In* Prasad MNV (ed), Heavy metal stress in plants. Springer, Berlin, Germany.
36. Vijayraghavan U, Company M, Abelson J. 1989. Isolation and characterization of pre-mRNA splicing mutants of Saccharomyces cerevisiae. Genes Dev 3:1206–1216. https://doi.org/10.1101/gad.3.8.1206.
37. Measday V, Moore L, Retnakaran R, Lee J, Donoviel M, Neiman AM, Andrews B. 1997. A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. Mol Cell Biol 17:1212–1223. https://doi.org/10.1128/MCB.17.3.1212.
38. Lin YS, Tsai SC, Lin HC, Hsiao CD, Wu SM. 2011. Changes of glycogen

metabolism in the gills and hepatic tissue of tilapia (Oreochromis mossambicus) during short-term Cd exposure. Comp Biochem Physiol C Toxicol Pharmacol 154:296–304. https://doi.org/10.1016/j.cbpc.2011.06.014.
39. Seu KJ, Cambrea LR, Everly RM, Hovis JS. 2006. Influence of lipid chemistry on membrane fluidity: tail and headgroup interactions. Biophys J 91:3727–3735. https://doi.org/10.1529/biophysj.106.084590.
40. Alexandre H, Rousseaux I, Charpentier C. 1994. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. FEMS Microbiol Lett 124:17–22. https://doi.org/10.1111/j.1574-6968.1994.tb07255.x.
41. Upchurch RG. 2008. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol Lett 30:967–977. https://doi.org/10.1007/s10529-008-9639-z.
42. De Palma M, Grillo S, Massarelli I, Costa A, Balogh G, Vigh L, Leone A. 2008. Regulation of desaturase gene expression, changes in membrane lipid composition and freezing tolerance in potato plants. Mol Breed 21:15–26. https://doi.org/10.1007/s11032-007-9105-y.
43. Thompson JE, Froese CD, Madey E, Smith MD, Hong Y. 1998. Lipid metabolism during plant senescence. Prog Lipid Res 37:119–141. https://doi.org/10.1016/S0163-7827(98)00006-X.
44. Tsaluchidu S, Puri BK. 2008. Fatty acids and oxidative stress. Ann Gen Psychiatry 7(Suppl 1):S86. https://doi.org/10.1186/1744-859X-7-S1-S86.
45. Yiin SJ, Lin TH. 1995. Lead-catalyzed peroxidation of essential unsaturated fatty acid. Biol Trace Elem Res 50:167–172. https://doi.org/10.1007/BF02789419.
46. Kwast KE, Burke PV, Staahl BT, Poyton RO. 1999. Oxygen sensing in yeast: evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. Proc Natl Acad Sci U S A 96:5446–5451. https://doi.org/10.1073/pnas.96.10.5446.
47. Lee K, Huang AH. 1994. Genes encoding oleosins in maize kernel of inbreds Mo17 and B73. Plant Mol Biol 26:1981–1987. https://doi.org/10.1007/BF00019508.
48. Zhou YE, Egeland GM, Meltzer SJ, Kubow S. 2009. The association of desaturase 9 and plasma fatty acid composition with insulin resistance-associated factors in female adolescents. Metabolism 58:158–166. https://doi.org/10.1016/j.metabol.2008.09.008.
49. Kudo N, Nakagawa Y, Waku K, Kawashima Y, Kozuka H. 1991. Prevention by zinc of cadmium inhibition of stearoyl-CoA desaturase in rat liver. Toxicology 68:133–142. https://doi.org/10.1016/0300-483X(91)90016-T.
50. Huang Z, Chen K, Zhang J, Li Y, Wang H, Cui D, Tang J, Liu Y, Shi X, Li W, Liu D, Chen R, Sucgang RS, Pan X. 2013. A functional variomics tool for discovering drug-resistance genes and drug targets. Cell Rep 3:577–585. https://doi.org/10.1016/j.celrep.2013.01.019.
51. Wang S, Kuang X, Fang Z, Huang Z, Shi P. 2014. Effect of oleic acid on the levels of eight metal ions in human hepatoma SMMC-7721 cells. Biol Trace Elem Res 159:445–450. https://doi.org/10.1007/s12011-014-0018-4.
52. Fang Z, Wang S, Du X, Shi P, Huang Z. 2014. Phosphatidate phosphatase-1 is functionally conserved in lipid synthesis and storage from human to yeast. Acta Biol Hung 65:481–492. https://doi.org/10.1556/ABiol.65.2014.4.11.
53. Prasitchoke P, Kaneko Y, Bamba T, Fukusaki E, Kobayashi A, Harashima S. 2007. Identification and characterization of a very long-chain fatty acid elongase gene in the methylotrophic yeast, Hansenula polymorpha. Gene 391:16–25. https://doi.org/10.1016/j.gene.2006.11.013.
54. Fang Z, Kuang X, Zhang Y, Shi P, Huang Z. 2015. A novel *HAC1*-based dual-luciferase reporter vector for detecting endoplasmic reticulum stress and unfolded protein response in yeast Saccharomyces cerevisiae. Plasmid 79:48–53. https://doi.org/10.1016/j.plasmid.2015.04.002.
55. Malik MA, Al-Thabaiti SA. 2012. Synthesis, structure optimization and antifungal screening of novel tetrazole ring bearing acyl-hydrazones. Int J Mol Sci 13:10880–10898. https://doi.org/10.3390/ijms130910880.
56. Yagi K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. Biochem Med 15:212–216. https://doi.org/10.1016/0006-2944(76)90049-1.