Multi-metal tolerance of DHHC palmitoyl transferase-like protein isolated from metal contaminated soil

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Abstract

The microbiota inhabiting in metal polluted environment develops strong defense mechanisms to combat pollution and sustain life. Investigating the functional genes of the eukaryotic microbiota inhabiting in these environments by using metatranscriptomics approach was the focus of this study. Size fractionated eukaryotic cDNA libraries (library A, < 0.5 kb, library B, 0.5-1.0 kb, and library C, > 1.0 kb) were constructed from RNA isolated from the metal contaminated soil. The library C was screened for Cadmium (Cd) tolerant genes by using Cd sensitive yeast mutant *ycf1*$^{\Delta}$ by functional complementation assay, which yielded various clones capable of growing in Cd amended media. One of the Cd tolerant clones, PLCg39 was selected because of its ability to grow at high concentrations of Cd. Sequence analysis of PLCg39 showed homology with DHHC palmitoyl transferases, which are responsible for addition of palmitoyl groups to proteins and usually possess metal coordination domains. The cDNA PLCg39 was able to confer tolerance to Cd-sensitive (*ycf1*$^{\Delta}$), Copper-sensitive (*cup1*$^{\Delta}$) and Zn-sensitive (*zrc1*$^{\Delta}$) yeast mutants when grown at different concentrations of Cd (40-100 µM), Cu (150-1000 µM) and Zn (10-13 mM), respectively. The DHHC mutant *akr1*$^{\Delta}$ transformed with PLCg39 rescued from the metal sensitivity indicating the role of DHHC palmitoyl transferase in metal tolerance. This study demonstrated that PLCg39 acts as a potential metal tolerant gene which could be used in bioremediation, biosensing and other biotechnological fields.

Introduction

Pollution of soil by potentially toxic metals commonly occurs through the addition and accumulation of contaminated waste water, mineral fertilizer and pesticide applications (Giller et al. 1998; Gulan et al. 2017; Fan et al. 2019). Soil contamination with these metals affect the soil microorganisms and disrupt their usual functioning like conformational modification of enzymes leading to their denaturation and inactivation (Bååth 1989; Chipasa 2003; Jaishankar et al. 2014) and production of free radicals due to disruption of metabolic reactions leading to oxidative stress (Dietz et al. 1999; Panda et al. 2003). However, few soil microorganisms are able to maintain normal cellular functions even at sublime levels of long-term exposure to toxic metals and sustain overall biomass comparable to normal unpolluted soils. As a measure to weaken the noxious effects of contaminating metals, many

microorganisms may have efficiently developed adaptation mechanisms. They usually employ extracellular precipitation mechanisms to reduce the entry of external metals like limited uptake, cell wall biosorption etc., or 'tolerate' metals by compartmentation within vacuoles, intracellular chelation assisted by metallothioneins and phytochelatins (Ji and Silver 1995). Potential molecular mechanisms have been identified in microorganisms and plants residing in metal contaminated soil (Clemens 2001; Dziewit and Drewniak 2016). However, comprehending these 'survival skills' faces a major challenge as a large chunk of the microbial population cannot be cultivated employing standard microbiological protocols. A large proportion of the uncultivable microbes are eukaryotes and they contribute to the rich gene pool comprising of novel biocatalysts participating in metal tolerance. Hence, identifying the genes and understanding their mechanistic roles in tolerating metal toxicity would enrich our knowledge and could also be implemented directly in various biotechnological applications (Marmeisse et al. 2017).

Functional metatranscriptomics is gaining an importance in identifying the potential eukaryotic genes directly from the environment involved in various biological activities. Metatranscriptomics starts with total RNA isolation from environmental sample and synthesizing cDNAs specifically from polyadenylated eukaryotic mRNA and subsequent preparation of cDNA library (Damon et al. 2012). This approach has been employed to probe complex microbiome of the gut of animals and humans (Jovel et al. 2016; Bikel et al. 2015), extreme environmental habitats (Tveit et al. 2014; Dopson and Holmes 2014), for different soil ecosystems as a tool to exploit the gene resources for industrially important enzymes (Kellner et al. 2011; Damon et al. 2011) and for metal resistance/tolerance (Mukherjee et al. 2019; Thakur et al. 2019; Mukherjee and Reddy 2020).

The present study reports the screening of eukaryotic cDNA library for genes involved in providing tolerance to metals from contaminated soil. Screening of cDNA library (library C) was performed utilizing functional complementation of metal sensitive phenotype of *S. cerevisiae* mutant. This led to the isolation of several genes providing tolerance to toxic metals including DHHC palmitoyl transferase-like protein, which demonstrated high tolerance towards toxic metals. DHHC is a domain containing protein defining a family of palmitoyl transferases, responsible for addition of a palmitoyl group to proteins for anchorage. Reports have suggested that the DHHC domain could fold as a zinc-finger domain and bind to zinc atoms, suggesting the metal binding potential of this protein (Montoro et al. 2009; Nurmemmedov and Thunnissen 2006). Very few reports are available about the role of palmitoyl transferase in metal tolerance. It has been reported that palmitoyl transferase (*AKR1*) act as a stress responsive gene and its mutation leads to hyper sensitivity towards the metal in yeast (Pagani et al. 2007). Gupta et al. (2018) identified DHHC palmitoyl transferase as a novel abiotic stress protein in *Triticum aestivum* through protein–protein interaction network analysis. Hence, not much work has been done to support the role of palmitoyl transferase in metal tolerance or abiotic stress. In this study, we have attempted to characterize the DHHC palmitoyl transferase-like protein with respect to its metal tolerance potential.

Material and methods

Sampling of soil

Soil samples were collected from Pierrelaye (PL) near northwest of Paris, France (49°1'45" N, 2°10'32"E). The experimental site was considered for vegetable gardening and the soil was irrigated with untreated wastewater from 1899 to 2002. This caused the accumulation of trace elements, which lead to multi-metal pollution characterized by ten times increase in the concentration of metals like lead, copper, zinc and cobalt. Crop production was thereby prohibited in the late 1990s (Phanthavongsa et al. 2017). This land was later converted into social forestry by planting poplar plantation in the year 2007 (Foulon et al. 2016; Lamy et al. 2006). Soil core samples were collected

from twenty different sites and composite sample was made by mixing equal volumes from each sample. Soil sample was sieved through a mesh of size 2 mm to remove plant parts and debris and transported in dry ice in sealed and sterile sampling bags to the laboratory where it was preserved at -80 °C. Physicochemical properties of the soil samples were analyzed for its pH, organic carbon (Walkley 1947); total nitrogen (Piper 1966) total phosphorous (Kitson and Mellon 1944) and available phosphorous (Olsen 1954). The soil sample was also analyzed for the presence of various metals through ICP-AES. Following are the physicochemical properties of the soil: pH 7.15; organic carbon 1.6%; available phosphorous 14.2 mg/kg; total phosphorous 291 mg/kg and total nitrogen 0.12%. Different toxic metals present in the soil samples were, Cadmium 2.5, Copper 64 and Zinc 385 mg/kg of soil.

Biological materials

Saccharomyces cerevisiae mutant strains used in this study were as follows: Copper-sensitive *cup1*$^{\Delta}$ where the copperthionein gene is omitted (Longo et al. 1996), cadmium-sensitive *ycf1*$^{\Delta}$, which is devoid of an ABC transporter gene YCF1 (Li et al. 1997) and zinc-sensitive *zrc1*$^{\Delta}$ with the deletion of a gene for transporter protein ZRC1 (Li and Kaplan 2001). These strains are derived from the parent wild type strain BY4741 (*MATα, his3*$^{\Delta}$*1, leu2Δ0, met15*$^{\Delta}$*0, ura3Δ0*), which was used as a metal tolerant reference strain. DTY3 (*cup1*^{s}) (*MATα, leu2-3, 112his3*$^{\Delta}$*1, trp1-1, ura3-50, gal1, CUP1*^{S}) having a single copy of *cup1* was also used as control. Palmitoyl transferase mutant strain *akr1*$^{\Delta}$ (BY4741; MAT*α*; ura3$^{\Delta}$0; leu2$^{\Delta}$0; his3$^{\Delta}$1; met15$^{\Delta}$0; YDR264c::kanMX4) was used for metal sensitivity experiments. Yeast strains were grown in synthetic defined media without uracil (SD-Ura), a minimal media supplemented with 2% glucose (Hi-media laboratories, India). For genetic transformation of bacteria, One Shot® *TOP10* Electrocomp™ *E. coli* (Thermofisher, USA) cells were used.

Size fractionation and cDNA library construction

Total RNA was isolated from soil samples by using PowerSoil® Total RNA Isolation Kit (Mo Bio laboratories, Carlsbad, CA). Integrity of the RNA was verified through gel electrophoresis and with Bioanalyzer 2100 (Agilent Technologies, USA). cDNAs were synthesized from total soil RNA with Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia). Size fractionation of cDNAs was performed as described in Yadav et al. (2014). Size fractions of cDNAs, A (<0.5 kb), B (0.5–1 kb) and C (>1 kb) were ligated downstream of PGK1 promoter of *S. cerevisiae* shuttle plasmid vector pFL61 modified with SfiA and SfiB restriction sites (Minet et al. 1992). These recombinant plasmids were introduced into DH10 β electrocompetant *E. coli* cells (One Shot® TOP10 Electrocomp™ *E. coli*, Invitrogen). More than 10^6 colonies growing on ampicillin-containing Luria agar plates (100 µg ampicillin per ml of medium) were pooled to represent each of the cDNA size libraries. The protocol used for isolation of metal tolerance genes through metatranscriptomic approach has been summarized in Fig. 1.

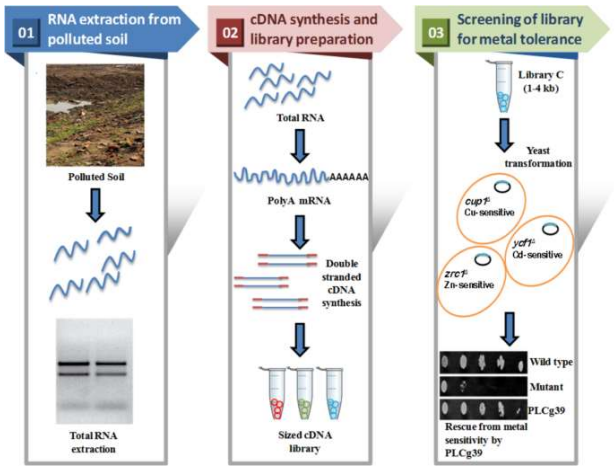


Fig. 1 Overview of the protocol used for isolation of genes involved in metal tolerance by metatranscriptomic approach

Screening of library C for genes involved in cadmium tolerance

Plasmid DNA from the pooled cells representing the cDNA library C (>1.0 kb) was extracted with QIAprep Spin Miniprep Kit (Qiagen, USA) and transformed into Cd-sensitive *ycf1*$^{\Delta}$ yeast mutant by lithium acetate method (Gietz et al. 1992) and were regenerated on SD-Ura medium amended with 40 µM Cd. The selected clones were treated with 5- Fluoroorotic Acid (5-FOA) to eliminate false positive colonies. One of the positive clones, PLCg39 was selected for further characterization based on its ability to tolerate high concentrations of Cd and other toxic metals such as Cu and Zn.

Sequence analysis of PLCg39

Plasmid DNA from yeast *ycf1*$^{\Delta}$/PLCg39 was isolated with Zymoprep yeast plasmid Miniprep II kit (Zymo Research) and sequenced the insert PLCg39 with vector specific primers NF (5'-CAGATCATCAAGGAAGTAATTATCTAC-3') and NR (5'-CAGAAAAGCA GGCT GGGAA GC-3'). Sequence homology search for PLCg39 was performed with BLASTX (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Homologous sequences were aligned by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/ clustalo/) and prediction of transmembrane helices and transmembrane domain of PLCg39 was carried out through HMMTOP server (http://www.enzim.hu/hmmtop/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/ TMHMM/), respectively. The databases and tools provided in CBS Prediction Servers (http://www.cbs.dtu.dk/services/) were used for glycosylation (NetOGlyc 4.0 Server) and phosphorylation sites prediction (NetPhos 3.1 Server). Phylogenetic analysis of DHHC palmitoyl transferase sequences from diverse taxons were reconstructed by using MEGA software (Kumar et al. 2016) with 1000 bootstrap replicates.

Metal tolerance of PLCg39

Genetic transformation of yeast mutants sensitive to Cu (*cup1*$^{\Delta}$), Cd (*ycf1*$^{\Delta}$) and Zn (*zrc1*$^{\Delta}$) was performed with plasmid pFL61 bearing PLCg39 to study the tolerance potential towards copper, cadmium and zinc respectively. The transformants were subjected to drop assay on SD-Ura agar supplemented with these metals. Yeast cultures *cup1*$^{\Delta}$/PLCg39, *ycf1*$^{\Delta}$/PLCg39 and *zrc1*$^{\Delta}$/PLCg39 were normalized to OD_{600} = 1.0 and serial dilutions were prepared (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in sterile distilled water. From these dilutions, five µl was dropped on SD-Ura medium agar plates containing 150 µM $CuSO_4$, 40 µM $CdCl_2$ and 10 mM $ZnCl_2$, respectively. The SD-Ura agar plates without metals served as controls. The growth assays of *cup1*$^{\Delta}$/PLCg39, *ycf1*$^{\Delta}$/PLCg39 and *zrc1*$^{\Delta}$/ PLCg39 was performed in presence of $CuSO_4$ (0-1000 µM), $CdCl_2$ (0-100 µM) and $ZnCl_2$ (0-13 mM) to study their tolerance levels to these metals. Empty vector pFL61 transformed yeast mutants and wild type BY4741 served as controls. The experiment was a 3 ×5 factorial design with yeast strains and concentrations of the metals arranged in a completely randomized block design with three replicates. The experiment consisted of three yeast inoculation treatments (*cup1*$^{\Delta}$/pFL61, *cup1*$^{\Delta}$/PLCg39 and BY4741) and five concentrations of $CuSO_4$ (0, 150, 300, 500 and 1000 µM $CuSO_4$). Similar experiments were performed for Cd and Zn using the respective yeast mutants. Percentage cell viability of a strain in presence of a metal was calculated by using the following formula Absorbanceat600nmwithmetalAbsorbanceat600nmwithoutmetal×100

The dose-response graphs were plotted and from the graphs IC_{50} value was calculated, which is the concentration required for inhibiting 50% of cell growth.

Further, the role of DHHC palmitoyl transferase in metal tolerance was confirmed by transforming PLCg39 to DHHC palmitoyl transferase mutant *akr1*$^{\Delta}$. Transformed *akr1*$^{\Delta}$/PLCg39 was screened on SD-Ura medium amended with 150 µM Cu. Drop out assay of *akr1*$^{\Delta}$/PLCg39 was performed in presence of 300 µM $CuSO_4$ as described above. For comparative analysis, the *akr1*$^{\Delta}$ mutant strain and BY4741 both

transformed with empty pFL61 were used. Additionally, BY4741 was also transformed with PLCg39 to investigate the collective role of the cDNA and the host DHHC palmitoyl transferase in providing metal tolerance to the wild type strain BY4741. The growth assay was performed according to the previously described protocol by supplementing 300 µM $CuSO_4$ into the media after 6 h and growth response was documented up to 36 h at an interval of 6 h.

Metal accumulation in yeast transformants

For accumulation studies, all yeast cells were allowed to grow overnight in SD-Ura medium till the OD_{600} = 1.0 was attained. Cells were harvested by centrifugation and re-suspended in sterile Milli-Q water. One percent inoculum was used to inoculate 50 ml of SD-Ura broth in 250 ml flasks and were incubated at 30 °C at 200 rpm. After 5 hours, various concentrations of toxic metals (Cu, Cd and Zn) were amended in the media similar to growth kinetics study. The experiment was a 3 × 4 factorial design with yeast strains and concentrations of the metals arranged in a completely randomized block design with three replicates. The experiment consisted of three yeast inoculation treatments (*cup1*$^{\Delta}$/pFL61, *cup1*$^{\Delta}$/PLCg39 and BY4741) and four concentrations of $CuSO_4$ (150, 300, 500 and 1000 µM $CuSO_4$). Similar experiments were carried out for Cd and Zn using the respective yeast mutants. Cells were harvested after 40 h of growth and washed 3 times with 10 mM EDTA solution. Final washing of cells was done with sterile deionized water to eliminate residual EDTA and dried for 24 h at 80 °C. A combination of HNO_3 and $HClO_4$ (ratio 2:1 v/v) was used to digest dry yeast biomass (Idera et al. 2015) and the metal content was determined through ICP-MS (Element XR, Thermo Fisher Scientific, Germany).

Statistical analyses

The statistical tests were carried out by using Graph Pad Prism (version 5.04) (GraphPad Prism, San Diego California, USA) software. Two-way analysis of variance was performed to examine the effect of various concentrations of metals on the cell viability and metal uptake of different yeast strains and their interactions.

Results and discussion

Screening of cDNA library for cadmium tolerance

The *ycf1*$^{\Delta}$ clones carrying the cDNA insets of library C (>1.0 kb) were rescued from Cd sensitivity when grown in presence of 40 µM of Cd, indicating the immense potential of genetic resource of the environmental cDNA possessing metal tolerance. One of such clones, PLCg39 was selected for further analysis based on its tolerance to high concentrations of Cd. Metatranscriptomic approach has been successfully used to isolate several genes of important ecological functions like organic matter degradation and transporter proteins in forest soil and industrially important enzymes (Damon et al. 2011; Kellner et al. 2011; Lehembre et al. 2013). Microbial activity and resistance towards extreme environments was studied extensively by using this approach (Auffret et al. 2015; Hua et al. 2015; Ma et al. 2019). Functional diversity has also been studied by metatranscriptomics in metal polluted soil ecosystem to unearth novel metal sequestering genes (Ziller et al. 2017).

Sequence analysis of PLCg39

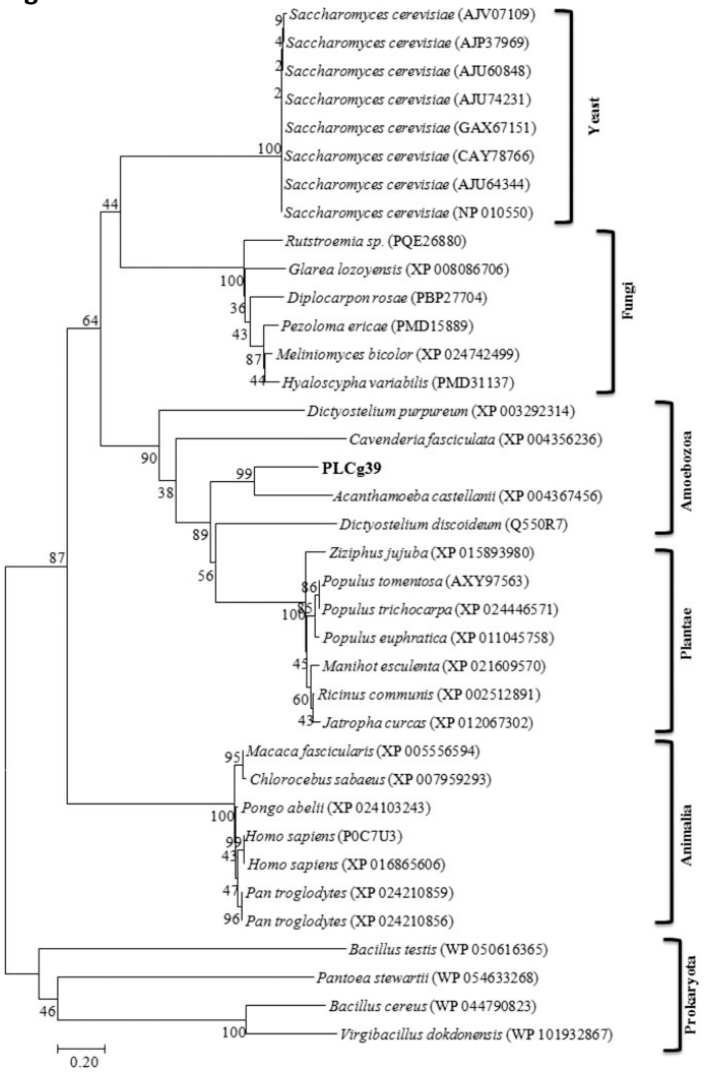
Sequence analysis revealed that PLCg39 is 1355 bp long with an open reading frame of 966 bp encoding a polypeptide of 321 amino acids. The molecular mass and pI of PLCg39 were predicted as 37.38 kDa and 9.39, respectively. BLASTX analysis showed a homology with DHHC zinc finger domain containing protein of an amoebozoan, *Acanthamoeba castellanii* with 58.97% identity. Sequence alignment with

other homologous DHHC zinc finger domains of the same phylum showed highly conserved zinc finger domain of PLCg39 (Fig. 2). The core DHHC motif of PLCg39 has a conserved region of 48 residues with a canonical sequence motif of $CX_2CX_9HCX_2CX_2CX_4DHHCX_5CX_4NX_3FX_4$. Other signature conserved regions are the DPG motif (Asp-Pro-Gly) adjacent to TMD2 and TTXE (Thr-Thr-Xaa-Glu) next to TMD4 (Montoro et al. 2009). Apart from the cysteine rich domain, PLCg39 has 3 CXXXC motifs (Supplementary Fig. S1). Tertiary structure homologous to PLCg39 was identified from PDB library through I-TASSER program. Significant PDB hits 6BMS, 6BML and 4JAD were identified based on Z-score and 6BMS (32.33 % identity) with highest Z-score was selected as the template to build the predicted models. COFACTOR deduced various ligand binding sites on the model with highest C-score or confidence score. Zinc binding residues for the predicted models were C^{129}, C^{132}, H^{142} and C^{149} (Supplementary Fig. S2). Sequence of PLCg39 obtained in this study was submitted to NCBI database under the accession number MK629535.

Fig. 2 Multiple sequence alignment of PLCg39 with members of the phylum Amoebozoa. Accession numbers are DHHC proteins of *Dictyostelium purpureum* (XP_003292314), *Acanthamoeba castellanii* str. *Neff* (XP_004367456), *Cavenderia fasciculate* (XP_004356236), *Dictyostelium discoideum* (Q550R7), *Acytostelium subglobosum* (XP_012756929), *Tieghemostelium lacteum* (KYQ90117), *Heterostelium album* (XP_020430454). The highly conserved zinc finger motif is highlighted in yellow and the DHHC motif in red

Phylogenetic analysis of DHHC palmitoyl transferase from diverse taxon have distinctively grouped into different lineages such as Yeast, Animalia, Fungi, Amoebozoa, Plantae and Prokaryota. PLCg39 was clustered with Amoebozoa lineage which possibly designates the origin of this eukaryotic gene from Amoeba (Fig. 3). Sequence analysis of PLCg39 revealed that it is polytopic protein having 4 transmembrane domains (TMD). DHHC domain containing family of proteins contains motif called DHHC-CRD (Asp-His-His-Cys cysteine-rich domain). This domain is responsible for the addition of a palmitoyl group to proteins as a part of post-translational modification in order to secure them to membranes (Putilina et al. 1999).

Fig. 3



Phylogenetic relation of the transcript PLCg39 with DHHC palmitoyltrasferase of other taxa. The tree has been constructed using the neighbor-joining method. Bootstrap values were obtained with 1000 replicates are indicated at the nodes. Kingdom names are denoted on the right side. GenBank Accession numbers are denoted in parentheses. Prokaryota DHHC palmitoyltrasferase sequences were included as outgroup. Branch lengths are proportional to evolutionary distances

Tolerance of PLCg39 towards toxic metals

The yeast mutants sensitive to Cu (*cup1*$^{\Delta}$), Cd (*ycf1*$^{\Delta}$) and Zn (*zrc1*$^{\Delta}$) transformed with PLCg39 demonstrated proficient growth in drop assay on SD-Ura media amended with 150 µM of Cu, 40 µM Cd and 10 mM Zn, respectively. The growth of the mutants transformed with empty vector pFL61 was inhibited at these concentrations. In general, the growth decreased with increase in cell dilutions in metal amended medium (Fig. 4). The ability to provide tolerance to various metal stresses by PLCg39, the transformed yeast mutants were grown in presence of different concentrations of metals (Cu, Cd and Zn). The transformant *cup1*$^{\Delta}$/PLCg39 showed high tolerance towards Cu in the medium compared to the wild type strain BY4741 and the *cup1*$^{\Delta}$ transformed with empty vector. The viability of the cells significantly decreased with increase in Cu concentration and was completely inhibited at 1000 µM of Cu (Fig. 5A). Significant variation was observed among the treatments for the two factors for its cell viability in presence of Cu including the interaction effect (Table 1). The viability of *ycf1*$^{\Delta}$/PLCg39 and BY4741 cells significantly decreased with increase in concentration of Cd and the *ycf1*$^{\Delta}$ cells

transformed with empty vector did not show the growth even at 40 µM Cd. Both *ycf1*$^{\Delta}$/PLCg39 and BY4741 cells showed similar tolerance at different concentrations of Cd (Fig. 5B). Significant variation among the treatments of yeast strains and concentrations of Cd along with their interaction effect was observed (Table 1). The viability of *zrc1*$^{\Delta}$ cells transformed with PLCg39 showed significantly higher growth than the wild type BY4741 in all concentrations tested. The growth of *zrc1*$^{\Delta}$ cells transformed with empty vector was completely inhibited even at 10 mM Zn (Fig. 5C). The IC_{50} values recorded for Cu by *cup1*$^{\Delta}$/PLCg39, BY4741/pFL61 and *cup1*$^{\Delta}$/pFL61 were 226.3 ± 18.9, 177.3 ± 22.6 and 23.8 ± 6.9 mM respectively (Fig. 6A). The *ycf1*$^{\Delta}$/PLCg39, BY4741.pFL61 and *ycf1*$^{\Delta}$/pFL61 cells showed IC_{50} values of 33.5 ± 6.2, 33.3 ± 3.4 and 10.2 ± 2.3 mM respectively for Cd (Fig. 6B). IC_{50} values of 11.2 ± 0.3, 10.7 ± 0.4 and 8.1 ± 0.8 µM were recorded for Zn by *zrc1*$^{\Delta}$/ PLCg39, BY4741/pFL61 and *zrc1*$^{\Delta}$/ pFL61 (Fig. 6C). These results indicated that yeast strains transformed with PLCg39 showed more tolerance to Cu and Cd compared to the wild type strain BY4741 and comparable with Zn.

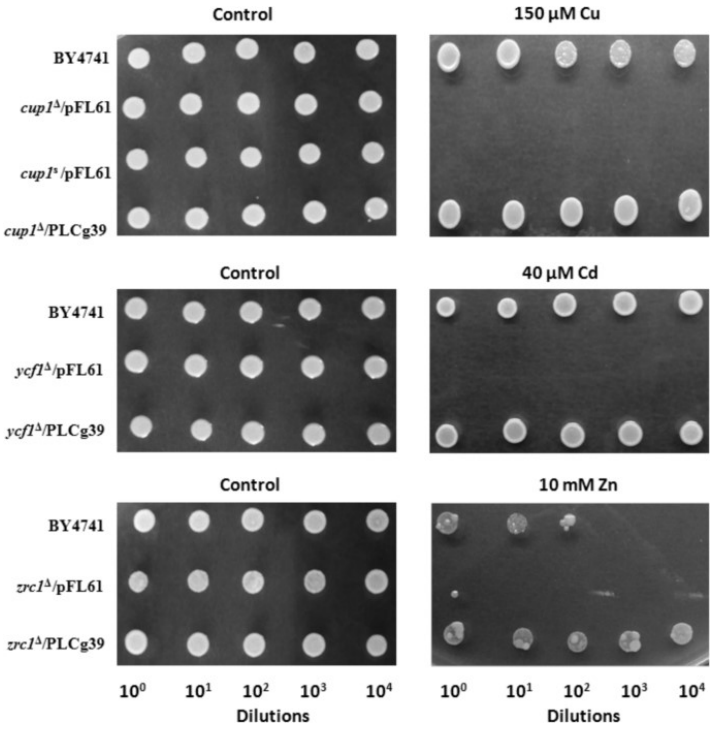
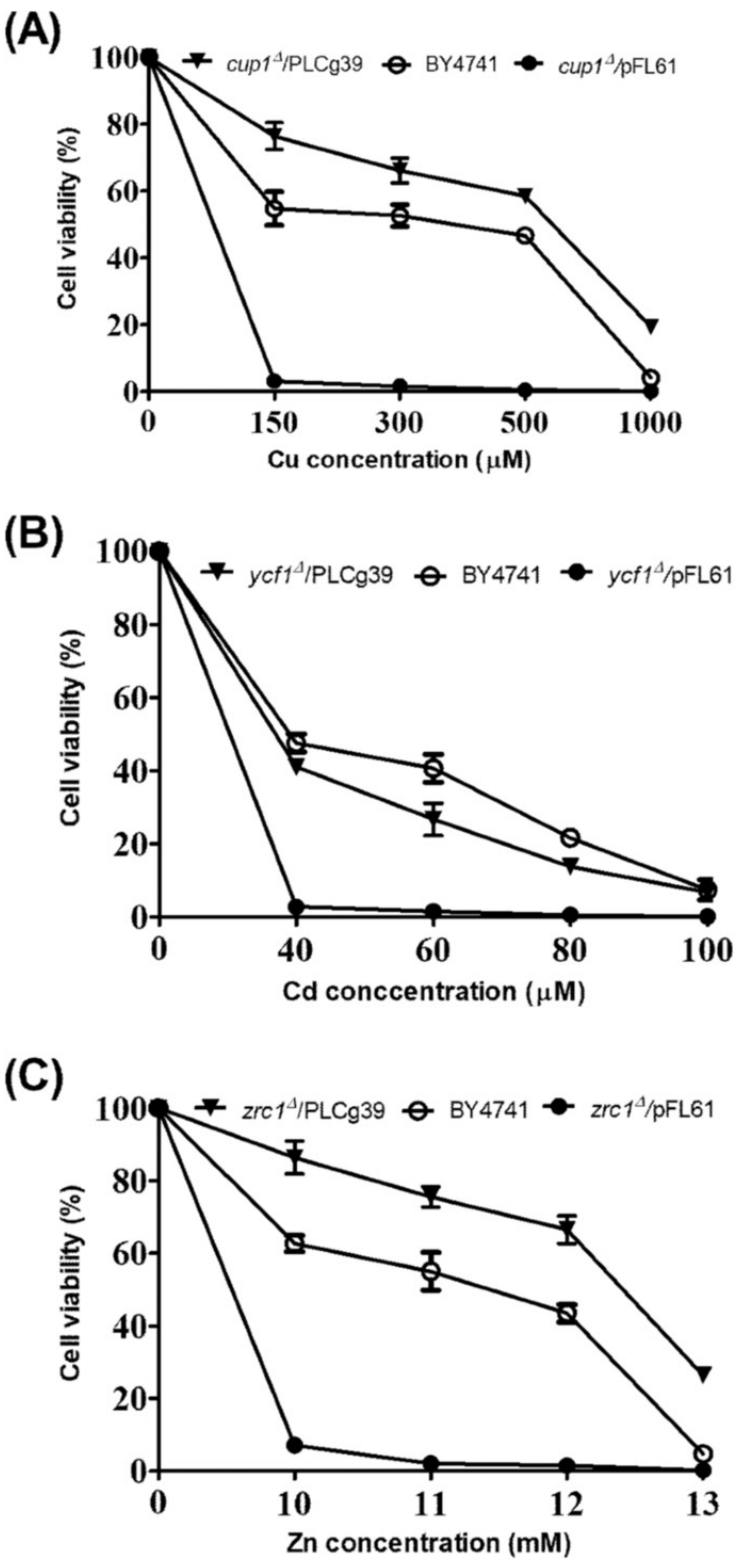


Fig. 4

Drop assay of PLCg39 transformed *Saccharomyces cerevisae* mutants, *cup1*$^{\Delta}$, *ycf1*$^{\Delta}$ and *zrc1*$^{\Delta}$ on SD-Ura agar. BY4741wild type and mutant strains transformed with empty vector pFL61 for positive and negative control. Serially diluted cultures were spotted on SD-Ura medium with or without metal supplement as indicated. Yeast strain *cup1*^{S} was also used as control

Fig. 5



Growth response expressed as percentage cell viability of (**A**) *cup1*$^{\Delta}$ (**B**) *ycf1*$^{\Delta}$ and (**C**) *zrc1*$^{\Delta}$ transformed with PLCg39 and wild type BY4741 transformed with Empty Vector pFL61 in SD-Ura broth supplemented with different metals at different concentrations

Table 1 The factorial ANOVA results indicating the effects of different concentrations of metals on cell viability and metal uptake by different yeast strains. The *p* values < 0.001 are highly significant

Variables	Yeast strains (YS)	Metal concentrations (MC)	YS x MC
Percent Cell viability			
df	2, 30	4, 30	8, 30
In presence of Cu	<0.001	<0.001	<0.001
In presence of Cd	<0.001	<0.001	<0.001
In presence of Zn	<0.001	<0.001	<0.001
Metal Uptake			
df	2, 24	3, 24	6, 24
Cu uptake	<0.001	<0.001	<0.001
Cd uptake	<0.001	<0.001	<0.001
Zn uptake	<0.001	<0.001	<0.001

1. df: degrees of freedom

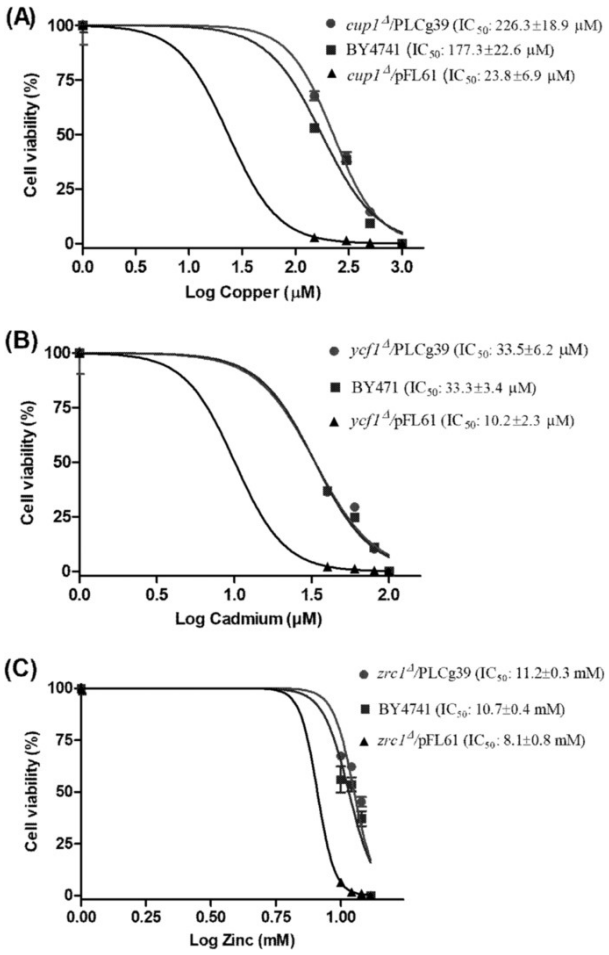


Fig. 6

Growth response and the IC_{50} values of (**A**) *cup1*$^{\Delta}$ (**B**) *ycf1*$^{\Delta}$ and (**C**) *zrc1*$^{\Delta}$ transformed with PLCg39 and wild type BY4741 transformed with Empty Vector pFL61 in SD-Ura broth supplemented with different metals at different concentrations analyzed through non-linear regression analysis

To confirm the role of DHHC palmitoyl transferase to rescue from metal sensitivity, the DHHC palmitoyl transferase mutant yeast strain *akr1*$^{\Delta}$ was transformed with PLCg39 and grown on SD-Ura medium with 300 µM $CuSO_4$. The transformant *akr1*$^{\Delta}$/PLCg39 was able to grow well when compared to the mutant and the growth was comparable with that of wild type BY4741, which clearly shows that DHHC palmitoyl transferase provide tolerance to metal stress (Fig. 7). To investigate the combined effect of

endogenous palmitoyl transferase and the introduced PLCg39, BY4741 strain was transformed with PLCg39 and allowed to grow in SD-Ura medium amended with 300 µM $CuSO_4$. In contrast to BY4741 bearing only empty pFL61 vector, the transformant BY4741/ PLCg39 showed more tolerance towards Cu stress. A significant variation was observed between the days and the yeast strain BY4741 transformed with and without PLCg39 and their interaction at $p < 0.001$. This indicated the synergistic role of PLCg39 and the endogenous DHHC protein in providing tolerance to Cu stress (Fig. 8).

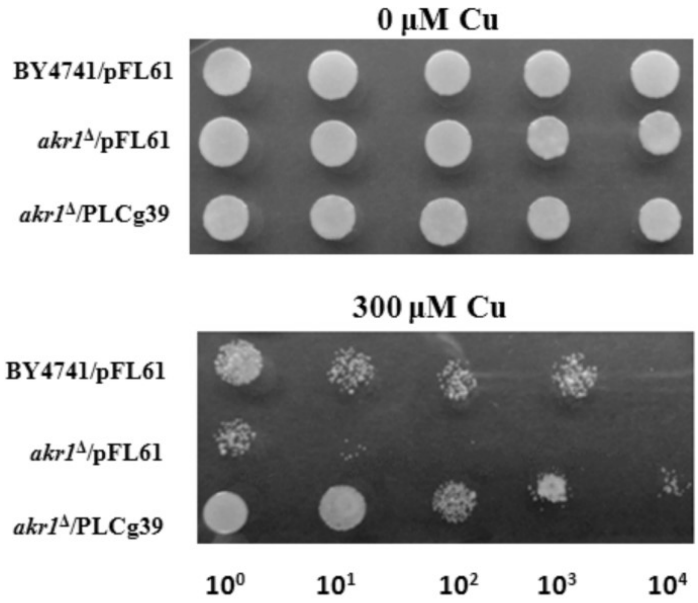


Fig. 7

Drop assay of PLCg39 transformed yeast mutant *akr1*$^{\Delta}$ on SD-Ura agar. BY4741wild type and mutant strain *akr1*$^{\Delta}$ were transformed with empty vector pFL61 for positive and negative control. Serially diluted cultures were spotted on SD-Ura medium with or without metal supplement as indicated

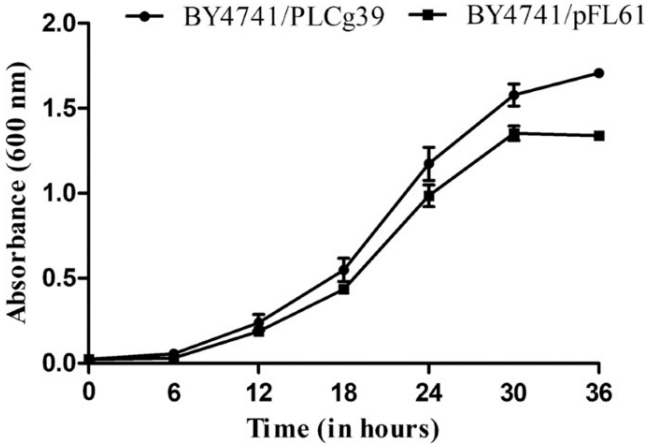


Fig. 8

Growth response of BY4741 transformed with PLCg39 and empty vector pFL61. Growth response was observed until 36 h at an interval of 6 h. Significant variation recorded between the yeast strains and the days and their interaction at $p < 0.001$

Accumulation of toxic metals

Accumulation of metals by the yeast mutants transformed with PLCg39 was studied by growing them at different concentrations of the metals. The *cup1*$^{\Delta}$ cells transformed with PLCg39 showed significantly higher accumulation of Cu at different concentrations compared to the wild type BY4741 as well as *cup1*$^{\Delta}$ cells transformed with empty vector in all concentrations of Cu. The accumulation of Cu levels decreased with increasing concentrations of Cu (Fig. 9A). Significant variation among the yeast strains and the concentrations of Cu including their interaction in relation to Cu accumulation was recorded (Table 1). The Cd content in *ycf1*$^{\Delta}$/PLCg39 was found to be higher compared to the wild type strain BY4741 in all the concentrations of Cd, highest being observed at 40 µM. The *ycf1*$^{\Delta}$ cells transformed with empty vector recorded lower or no Cd content in the cells due to their growth inhibition at all the concentrations of Cd (Fig. 9B). Significant variation among the yeast strains and Cd

concentrations along with their interaction observed in respect to Cd uptake (Table 1). A significant amount of Zn accumulation was observed in case of *zrc1*$^{\Delta}$/ PLCg39 when compared to the wild type BY471. Maximum Zn accumulation was recorded at 10 mM than the other concentrations tested. The Zn content significantly affected in the wild type strain BY4741 as well as *zrc1*$^{\Delta}$ transformed with empty vector (Fig. 9C). A significant variation was observed among the yeast strains and Zn concentrations and their interaction (Table 1).

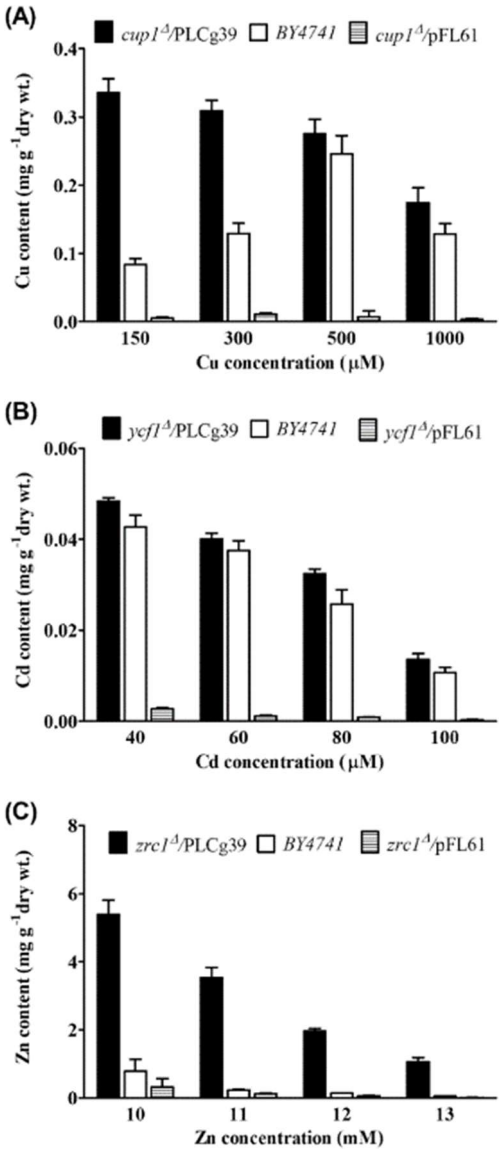


Fig. 9

Effect of different concentration of (**A**) Copper (**B**) Cadmium and (**C**) Zinc on metal content by PLCg39 when compared to wild type BY4741 and yeast mutants transformed with empty pFL61 after 40 h of growth

DHHC proteins play an important role as protein acyltransferases (PAT) as vast majority of proteins are fatty acylated as a form of post-translational modifications. These proteins are primarily annotated containing a zinc finger domain on the basis of conserved pattern of cysteine and histidine residues. Studies have demonstrated that any mutation of the conserved region resulted in the loss of function of the protein as the zinc binding ability is lost. It has also been reported that a selective pressure is essential for maintenance of the zinc finger pattern in DHHC protein (Gottlieb and Linder 2017). Stoichiometric quantification has proved that there are two zinc binding sites per DHHC protein. Hence, binding of zinc has been associated with structural rather than functional role. There are limited reports on the functional role of a DHHC protein in providing metal tolerance to eukaryotic cells. However, the role of zinc finger has been studied extensively as a mediator in metal homeostasis. The outcome of a range of abiotic stresses has been studied and found to be positively correlated with the increase in induction of zinc finger gene (Dai et al. 2007). Responses to metal stresses have been

studied in zinc fingers and metals like cadmium and copper, where increase of zinc finger transcripts and conferring resistance to these metals were reported (Dixit and Dhankher 2011; Singh et al. 2016; Sugano et al. 2003). Chen et al. (2016) reported the role of zinc finger transcriptional factor as a regulator for cadmium tolerance. Overexpression of a zinc finger from rice conferred tolerance to a wide range of abiotic stresses including toxic metals in transgenic tobacco plants (Mukhopadhyay et al. 2004).

DHHC palmitoyl transferase activity of the mutant yeast *akr1*$^{\Delta}$ was restored by PLCg39. This mutant lacks a functional DHHC palmitoyl transferase, which makes it lose protein palmitoylation activity and hence form abnormality in cell morphology and becomes susceptible towards heat, toxic metals, hyperosmotic stress, chemical compound treatment etc., and causes overall decrease in vegetative growth (Dudley et al. 2005; Enyenihi and Saunders 2003; Pagani et al. 2007; van Bakel et al. 2005; Zhang et al. 2002). It has been reported that the gene *AKR1* is important for genetic requirement for cadmium tolerance in yeast and contributed to the regulatory responses in presence of toxic concentrations of Cd^{2+} (Serero et al. 2008). Pagani et al. (2007) reported that AKR1 is a major stress responsive gene and mutation causes hyper sensitivity towards the metal. This encouraged us to test mutant *akr1*$^{\Delta}$ in presence of divalent cation Cu^{2+} and tested the response by functionally complementing with cDNA PLCg39.

The accumulation of toxic metals by PLCg39 may be attributed to the DHHC-CRD containing a zinc finger. As mentioned earlier, the binding of zinc is necessary to maintain the functional role of the protein. However, the extent of metal accumulation in eukaryotic cells by a DHHC protein has not been studied earlier. Zinc fingers are associated with metal binding and are found to be flexible with metals other than zinc. Kluska et al. (2018) reported that zinc fingers form stable complexes with cobalt, thus proving flexible coordination sites. The binding of cadmium and copper to zinc fingers was also studied as a replacement to zinc ions to regenerate the DNA binding activity of the zinc finger (Predki and Sarkar 1994). In general, the zinc finger allows the binding of toxic metals which could explain the ability of PLCg39 for intracellular accumulation of toxic levels of metals. In recent study by Thakur et al. (2018), an environment transcript showing homology with a zinc finger fusion protein ubiquitin containing the conserved cysteine and histidine residues demonstrated significant tolerance towards toxic concentrations of Cu, Cd and Zn. The present study has demonstrated the function of a DHHC protein containing a zinc motif which is able to tolerate toxic levels as well as accumulate toxic metals intracellularly.

The expression of transcript PLCg39 in *Saccharomyces cerevisiae* mutants resulted in tolerance towards different toxic metals in the order: Zn > Cu > Cd. The primary role of zinc fingers is the binding of the metal Zn which provides a structural stability to the protein. A significant percentage of zinc binding proteins found in *S. cerevisiae* are believed to have a role in the cell's response to stress, as the yeast cells rely on this group of regulators to manage environmental stress (Hartwig 2001). The abiotic stress imposed by toxic metals also induces biotic stress tolerance in organisms. This cross-tolerance is a result of interactions between the pathways of biotic and abiotic stress tolerance mechanisms. It has been observed that aluminum stress in potato roots resulted in increased defense response against disease causing *Phytophthora infestans* through a series of up-regulation of defense genes (Arasimowicz-Jelonek et al. 2014). Similar results were observed in Pepper (*Capsicum annuum* L.) plants where copper stress caused up-regulation of peroxidase genes and accumulation of copper lead to biocidal effects in the plant when exposed to *Verticillium dahlia*. However, the authors observed a decrease in chitinase activity which, when coupled with an increase in peroxidase activity, helps in the deposition of cell wall strengthening material. This acts as a primary barrier to biotic stresses (Chmielowska et al. 2010; Fones et al. 2010). A detailed review by Morkunas et al. (2018) on how metal stress affects biotic response in plants may be referred further.

Another probable explanation for the accumulation of these metals could be the presence of three CXXXC potential metal binding motifs in PLCg39. Various metallothioneins are found to contain CXXXC motifs and thus presumed to have the capability to ligate divalent metals (Yang et al. 2014). Sequestration of copper by a cytochrome c oxidase has a highly conserved CXXXC domain which is a putative copper binding motif (Jaksch et al. 2001; Rosenzweig 2002). Metal ion binding motifs having a conserved Cys-X_{2-4}-Cys domain have been identified as effective chemical sensors for detection of toxic metals and metalloids like Cadmium and Arsenic in aqueous solutions. This renders the use of such proteins/peptides for developing effective biosensing strategies (Joshi et al. 2009).

The potential of cDNA PLCg39 to tolerate toxic concentrations of toxic metals was quite evident from the experimental observations. The translated protein had the characteristics of a DHHC palmitoyl transferase. This protein has an important function as far as post-translational modification of other proteins is concerned. Thus, the approach of metatranscriptomics can reveal diversity of functions related to stress tolerance, when applied to complex environments like polluted soil. Heterologous expression of the eukaryotic gene proved that it could be utilized for a number of biotechnological applications like revegetation of metal contaminated sites, a biosensor for contamination studies and sequestration of toxic metals leading to effective bioremediation strategies.

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