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tRNA Wobble Base Modifications and Boric Acid Resistance in Yeast: Boron-Resistant Deletion Mutants Induce the General Amino Acid Control Mechanism and Activate Boron Efflux

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Abstract—Boric acid is essential for plants and has many vital roles in animals and microorganisms. However, its high doses are toxic to all organisms. We previously screened yeast deletion collections to identify boric acid-resistant and susceptible mutants to identify genes that play a role in boron tolerance. Here, we analyzed boron resistant mutants (*elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ) for their abilities to modulate the general amino acid control system (GAAC) and to induce boron efflux pump *ATR1*. The mutants analyzed in this study lack the genes that play roles in tRNA Wobble base modifications. We found that all of the boron resistant mutants activated Gcn4-dependent reporter gene activity and increased the transcript level of the *ATR1* gene. Additionally, boron resistant cells accumulated less boric acid in their cytoplasm compared to the wild type cells upon boron exposure. Thus, our findings suggested that loss of wobble base modifications in tRNA leads to GAAC activation and *ATR1* induction, which in turn reduced intracellular boron levels and caused boron resistance.

Keywords: boron, boric acid, tRNA, Wobble base, boron resistance, elongator complex, yeast, general amino acid control

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INTRODUCTION

Boron (B) has been known for about a century as a necessary metalloid for the growth and development of plants [1]. It has been shown to play different important metabolic and structural roles in cells, one of which is acting as a crosslinker among pectin rhamnogalacturonan II in plant cell walls [2–4]. However, molecular mechanisms underlying many of the roles in which boron takes part are literally unknown.

Recent studies showed that boron is an essential micronutrient for a broad spectrum of organisms, from bacteria to animals although the required amounts of boron differ among these organisms [5–10]. It can be toxic when present at high concentrations. However, the physiological basis of this toxicity is still unclear. Boron transport is noteworthy in this regard, and many genes playing roles in boron transport and tolerance have been identified in plants and yeast so far. *Arabidopsis thaliana BOR1* gene was identified as the first boron transporter gene, its homologs have been identified in many organisms [11–14].

Yeast *Saccharomyces cerevisiae* has been widely used to understand the mechanisms by which cells respond to various abiotic stresses, including copper, iron, zinc metals. To characterize plant boron tolerance genes, researchers used yeast as a model system [15–19]. Yeast Atr1 functions as a boron efflux pump providing boron resistance to the cells [15]. The expression of *ATR1* and the genes related to amino acid biosynthesis is regulated by the transcription factor Gcn4 in response to boric acid treatment. It has also been shown that boron activates Gcn2 kinase and leads to the phosphorylation of eIF2α, which then inhibits protein synthesis [20]. Genome-wide screening studies in yeast have also revealed some new genes related to boron metabolism. A haploid yeast deletion library was screened for this purpose, and 6 mutants have been identified as boron resistant mutants [21]. All of these mutants (*elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ) were deficient in Wobble base modifications in tRNA.

In this study, the genes deletion of which confer boron resistance to the yeast cells were investigated to underline their mechanisms for boron tolerance. We found that all boron resistant mutants constitutively

induced the GAAC path and *ATR1* gene expression, and thus, lowered their intracellular boron concentrations to gain resistance to toxic levels of boron.

EXPERIMENTAL

Yeast growth, media and transformation. Wild-type *Saccharomyces cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*; EUROSCARF) and its isogenic deletion mutants were obtained from the yeast deletion library. Cells were grown in either YPD (2% glucose, 1% yeast extract, 2% peptone and 2% agar for solid media) or YNB (yeast nitrogen base) (2% glucose, 0.67% yeast nitrogen base without amino acids and 2% agar) media supplemented with required amino acids and bases. Yeast transformations were performed using the LiAc method [22].

Boron Tolerance Assays. Boric acid was used as the boron source in the assays. YPD or YNB agar plates with different concentrations of boric acid were prepared. Overnight cultures were diluted to an optical density of 0.2 at 600 nm and then serially diluted to 0.02, 0.002, and 0.0002. A 5 μL aliquot of each dilution was spotted on these plates and incubated for 3 days at 30°C, and plates were photographed.

RNA isolation and real-time PCR analyses. Total RNA was isolated from boron-treated and untreated samples using RNA Isolation Kit (Invitrogen). Genomic DNA traces were removed by DNAse treatment (Fermentas). Complementary DNA (cDNA) from total mRNA was obtained using the First Strand cDNA Synthesis Kit (Fermentas). The cDNA was used as a template for amplifying a 186 bp fragment for the *ATR1* gene, and a 103 bp fragment for the internal control *ACT1* gene. Triplicate real-time PCR analyses were performed with the IQ5 Real-Time PCR System (Bio-Rad) using Fermentas MaximaTM SYBR Green qPCR Master Mix (2X). The primers used for the amplification of *ATR1* were ATR1F (5'-ACGCGTATAGCATAGCCGCTTTCA-3') and ATR1B (5'-TGTAAGCCTGGTTCCAACCCGATA-3'), the primers used for the amplification of *ACT1* were ACT1F (5'-ACGTTCCAGCCTTCTACGTTTCCA-3') and ACT1B (5'-ACGTGAGTAACACCATCACCGGAA-3'). The conditions of PCR amplification for the genes were as follows: 40 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s.

Molecular cloning. Gateway Cloning System (Invitrogen) was used for the cloning experiments. Wild type alleles of related genes were amplified using High Fidelity Enzyme (Fermentas) from yeast genomic DNA. The amplified fragments were first cloned into the pDONR vector using BP ClonaseTM II enzyme (Invitrogen) and then moved to LR clonase reaction. This reaction was performed with isolated entry clones, pAG426GPD expression vector, and LR ClonaseTM II enzyme mixture (Invitrogen). Isolated plasmids were verified by sequence analyses, and then yeast cells were transformed with the empty vector or with a gene expression construct and selected for uracil prototrophy.

β-Galactosidase activity assays. Wild type and isogenic yeast mutant cells were transformed with the p180 plasmid, which carries the *GCN4-lacZ* gene [23]. Overnight cultures of the wild-type cells in liquid YNB without uracil were diluted and after two hours of incubation, cultures were split into two flasks with or without 20 mM boric acid. After 3 hours of growth in the presence of boron, 500 μL of cell solution, at an optical density between 0.2 and 0.6 at 600 nm, was collected and homogenized in 400 μL of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol and 0.2% N-lauryl sarcosine). After 30 min preincubation at 30°C, 150 μL of Z-buffer with 4 mg/mL ONPG (o-nitrophenyl-b-D galactopyranoside) (Sigma) was added to the suspensions and the resulting solutions were incubated at 30°C for half an hour. The reaction was stopped by adding 400 μL 1.5 M Na_2CO_3. After centrifugation for 1 min, supernatants were taken and their absorbance values were determined at 420 nm and normalized by the OD_{600} values of cells.

Determination of intracellular boron concentrations. Exponentially growing boron-resistant mutant cells were treated with 50 mM boric acid for 1 hour or not and harvested by centrifugation. The cells were washed with distilled water and disrupted by using lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 7.6, 1 mM EDTA), glass beads, and vortex. The supernatants were used for further analyses. The boron concentrations of diluted cell extracts were determined using the Boron Cell Test Kit (Merck). The method determines photometrically the color change in the environment and has high measurement sensitivity (0.05 mg boron/liter). The obtained absorbance values were normalized by the values of corresponding cell cultures. The experiments were repeated three times.

RESULTS

Expression of Wobble Base Modification Genes and Boron Tolerance Levels

Wild type yeast can grow in the presence of 75 mM boric acid [15, 16]. The tRNA wobble base modification mutants *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ could tolerate up to 125 mM boric acid (Fig. 1a). We first cloned and overexpressed these genes in wild-type cells to find out whether their activation alters cell's response to boron. As Fig. 1b shows, overexpression of these genes did not change their response to the boron stress. Thus, their deletion rather than activation was necessary for boron tolerance.

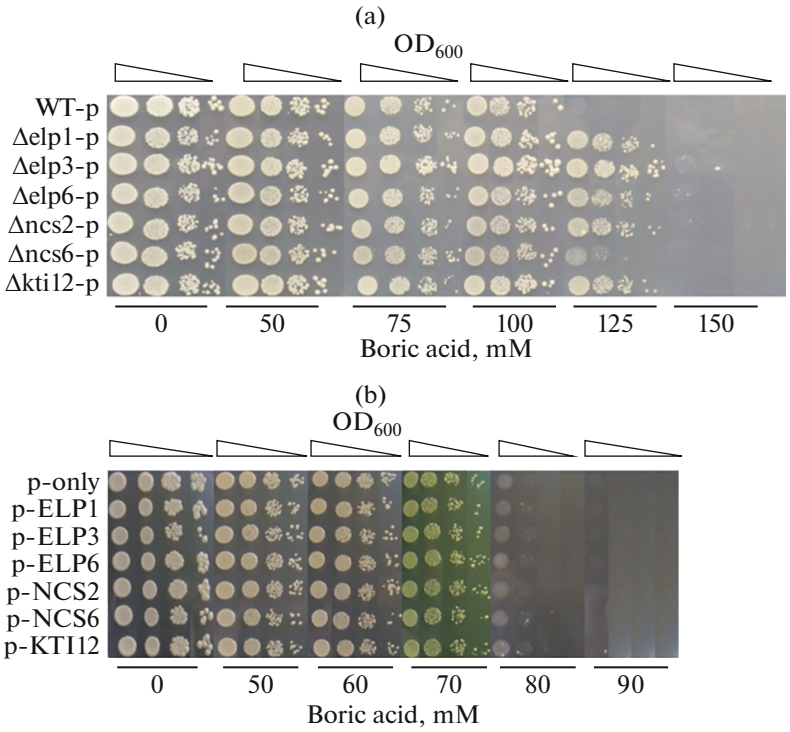

Fig. 1. Boric acid tolerance levels. (a) Wild type cells and boron resistant mutants were transformed with an empty vector (p426). (b) Wild-type cells were transformed with the plasmids containing *ELP1*, *ELP3*, *ELP6*, *NCS2*, *NCS6*, *KTI12* genes.

Gcn4 Activity and ATR1 Expression in the Mutant Cells

In order to understand the mechanisms by which they gain tolerance to boron, we analyzed the status of the general amino acid control mechanism (GAAC) and *ATR1* gene in the mutants. Only a few genes have been suggested to play a role in yeast boron tolerance and the roles of the ones other than *ATR1* are uncertain. *ATR1* is regulated by the Gcn4 transcription factor, which is the master regulator of amino acid biosynthesis genes [15, 20]. Defects in processing the wobble base of tRNA are known to induce *GCN4* translation [24]. Therefore, we focused on the status of *GCN4* transcription factor and *ATR1* gene in the mutants.

First, using a plasmid-based Gcn4-LacZ reporter system [23], we determined relative Gcn4 activity in the mutant cells along with the wild-type cells (Fig. 2a). The Gcn4 activity was found to be higher in all mutants changing from 1.4- to 3.6-fold when compared to that of the wild-type cells. When cells were treated with boron, Gcn4 activity was further elevated in the mutants. Thus, the tRNA wobble base modification mutants had an activated GAAC mechanism. Induction of *ATR1* boron efflux pump is one of the outcomes of GAAC activation, in fact, Gcn4 transcription factor simultaneously upregulates the expression of amino acid biosynthetic genes and *ATR1* gene [15]. Hence we monitored the transcript level of *ATR1* in the mutants. As seen in Fig. 2b, cells showed higher level expression (from 5 to 20-fold) of ATR1 as compared to the wild-type cells. Obviously, our boron resistant mutants had constitutively high Gcn4 activity and *ATR1* expression. This might be the reason for the boric acid tolerance of corresponding mutants. The high expression levels of *ATR1* in these mutants can result in the efflux of high amounts of boric acid so that the mutants might have low levels of boron in the cell. Intracellular boron levels were measured and found lower in the mutants that are resistant to boron when compared to the wild type (Fig. 2c).

Overexpression of ATR1 from a Plasmid Provides Further Boron Resistance to the Mutants

To test if an extra boron resistance could be achieved by further expression of *ATR1* gene in boron resistant mutants, we transformed the cells with a high copy number plasmid harboring the *ATR1* gene. We observed that *ATR1* overexpressing *elp3*Δ and *ncs6*Δ transformants could tolerate up to 350 mM boric acid, while the other transformants showed a wild-type-like growth pattern (Fig. 3).

The Wooble Base Modification Mutants Are Not Osmotolerant

The high-osmolarity glycerol (HOG) pathway has been suggested to be a part of the response to boric acid in yeast [25]. To find out whether the mutants

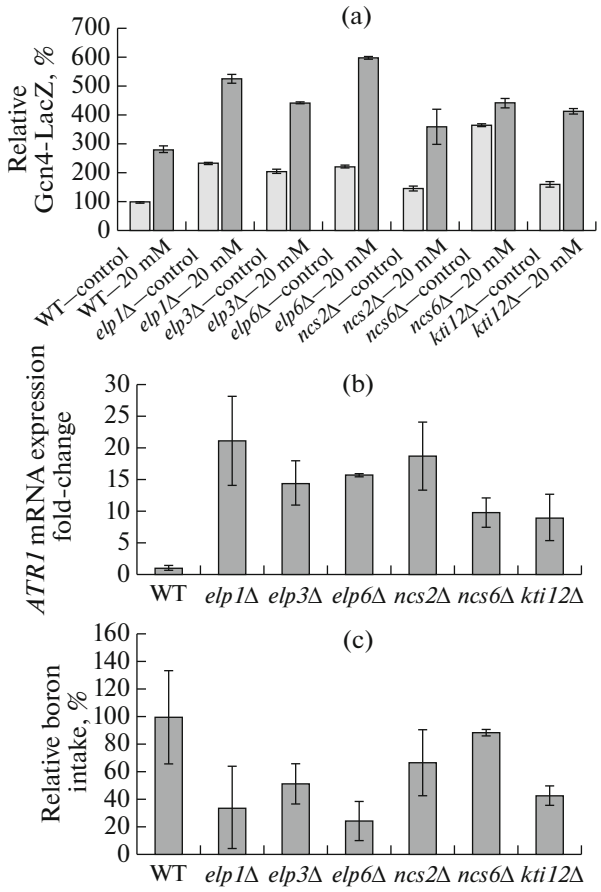

Fig. 2. Determination of Gcn4 activity, *ATR1* mRNA levels and intracellular boron concentrations of the mutants. (a) Wild type and boron resistant mutants were transformed with a plasmid containing *GCN4-LacZ* reporter gene. Logarithmically growing cultures were treated with 20mM boric acid for 3 hours or not. β-Galactosidase assay was performed as indicated in Experimental. (b) *ATR1* gene expression was determined by real-time PCR analyses in wild type and boron resistant mutants. The yeast actin gene was used as the internal control. (c) Intracellular boron levels of wild type and mutants that were exposed to 50 mM boric acid for 1 h. Bars represent the mean values of triplicate analyses.

show tolerance to salt and sorbitol, we spotted them onto agar plates containing different amounts of salt and sorbitol. There were no differences between the mutants and the wild type cells in terms of their salt and sorbitol tolerances (Figs. 4a, 4b) which suggest that boron resistance phenotype of the mutants is not a result of the general stress response and it is rather specific to boron.

DISCUSSION

We showed that the Gcn4 transcription factor and Atr1 boron efflux pump are activated in the yeast mutants deficient in the wobble base modifications of tRNA. The tRNAs are essential components of protein synthesis machinery and undergo many post-transcriptional changes. Hypo-modification of the wobble position seems to be very relevant to translational regulation. Especially, U34, which reads the third position in codons, is a frequent target of modifications that modulates wobble base pairing and persuading codon recognition.

In yeast, many tRNAs are modified at U34 and this process is orchestrated by more than two dozens of genes including the elongator complex (Elp1–Elp6), killer toxin-insensitive genes (Kti11–Kti14) and uridine thiolating ligases (Ncs2 and Ncs4) [26, 27]. The mutants that we analyzed in this work are all related to U34 modifications and their absence was reported to cause diverse phenotypes in response to different types of stress conditions [28, 29]. Under some circumstances the lack of tRNA modification may become beneficial for the cells; for example, thiolation deficient cells may confer resistance to endoplasmic reticulum stress [30]. Similarly, we showed that absence of U34 modifications provides boron resistance.

Our data and previous findings suggest that alterations in the tRNA modification pattern at U34 mediate cellular response to different stress conditions. What happens to protein synthesis machinery in the absence of tRNA modification is relatively known and it involves slow translation, recoding and synthesis of incorrect proteins [27]. However, the mechanism of

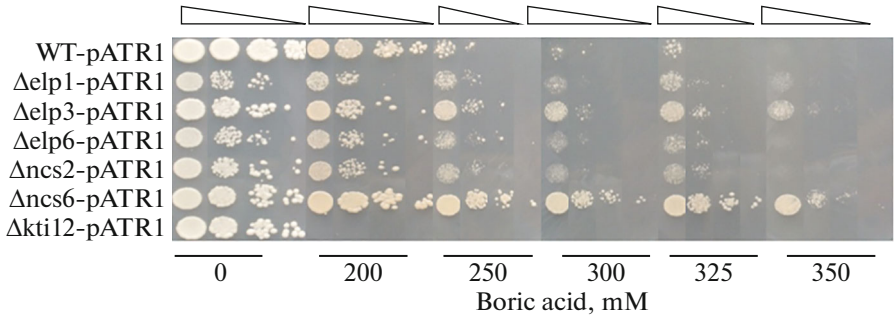

Fig. 3. Overexpression of *ATR1* boron efflux pump in boron resistant mutants increases boron tolerance. Each mutant was transformed with pATR1 plasmid and exposed to indicated concentrations of boric acid.

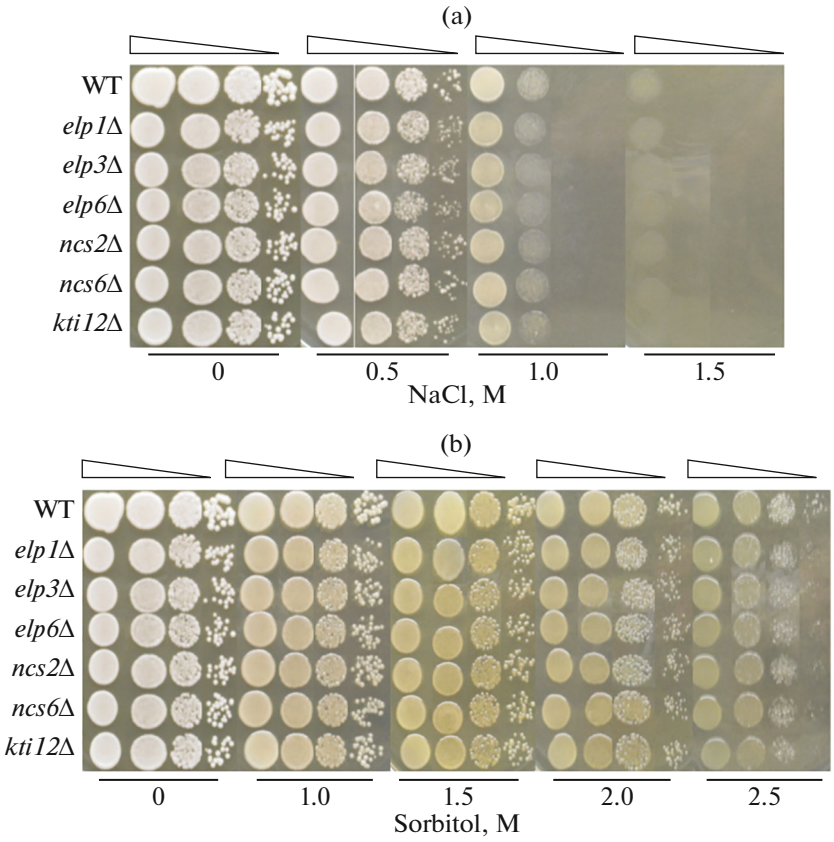

Fig. 4. Osmotic stress tolerances of boron resistant yeast mutants. Wild type and yeast mutants were grown to logarithmic phase and serially diluted to an optical density of 0.2, 0.02, 0.002, and 0.0002 at 600 nm. 5 μL of each dilution was spotted onto YPD media containing the indicated amount of sodium chloride (a) and sorbitol (b). The plates were incubated at 30°C for 3 days and photographed.

how the tRNA modifications regulate stress response is not clear for most situations.

Boron stress tolerance mechanism of *elp1*Δ, *elp3*Δ, *elp6*Δ, *ncs2*Δ, *ncs6*Δ and *kti12*Δ mutants could be explained by the induction of Atr1 boron efflux pump through the Gcn4 transcription factor. There could be additional mechanisms; for example, tRNAs in these mutants might escape from cleavage. As a part of stress response mechanism, tRNAs can be cleaved by specific nucleases, as is the case for amino acid starvation which results in tRNA cleavage in *Tetrahymena thermophila* and *Aspergillus fumigatus* [31, 32]. Oxidative stress can also result in tRNA cleavage in the anticodon loop in *Saccharomyces cerevisiae* [33, 34]. The mechanisms and functions of tRNA cleavage in different stress conditions are not clear. However, they may have regulatory roles during the stress response.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest.

This article does not contain any research involving humans or animals as subjects of research.

ADDITIONAL INFORMATION

The text was submitted by the author(s) in English.

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