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PKA-dependent remodeling of trehalose metabolism under boric acid stress

Tülay TURGUT GENÇ *

Department of Biology, Science Faculty, Çanakkale Onsekiz Mart University, 17020, Çanakkale, Türkiye.

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Abstract

Trehalose metabolism is essential for stress protection and metabolic adaptation in *Saccharomyces cerevisiae*. Boric acid, a micronutrient that becomes toxic at elevated levels, is known to disrupt metabolic balance and multiple stress-response pathways including those controlled by PKA. The impact of this compound on trehalose regulation, however, remains unclear. To determine how PKA activity influences trehalose regulation under boron stress, we analyzed *NTH1* promoter activity and trehalose accumulation in a wild type and a PKA-hyperactive mutant in yeast cells across three physiological phases: early exponential growth, extended exposure, and the post-diauxic state. In the wild type, boric acid suppressed *NTH1* promoter activity in the early phase but markedly increased trehalose levels, indicating enhanced synthesis during limited trehalase expression. The PKA-hyperactive mutant showed almost no induction of trehalose in this phase, revealing a loss of normal stress-responsive trehalose accumulation. During extended exposure, boric acid reduced trehalose only in wild type, suggesting impaired synthesis after prolonged stress; the mutant again showed minimal change. In the post-diauxic phase, boric acid triggered a regulatory shift in the wild type, with high trehalose and modest *NTH1* activation, while the mutant remained unresponsive. Together, these results demonstrated that boric acid modifies trehalose regulation in a strongly PKA-dependent and phase-specific manner, and that elevated PKA activity restricts metabolic flexibility and prevents appropriate trehalose remodeling under boron stress.

Keywords: Boric acid; Trehalose metabolism; *NTH1*; PKA-signalling; *Saccharomyces cerevisiae*

1. Introduction

Trehalose is a central reserve carbohydrate in *Saccharomyces cerevisiae* that plays a crucial role in stress tolerance, metabolic remodeling, and cellular survival. Its synthesis and degradation are tightly coordinated through the activities of the trehalose-6-phosphate synthase/phosphatase (TPS) complex (Tps1, Tps2, Tps3) and the neutral trehalase Nth1 [1-3]. Trehalose functions not only as a carbon and energy store but also as a highly-effective chemical chaperone that stabilizes proteins and membranes under diverse stress conditions, including heat, osmotic shock, oxidative imbalance, and nutrient depletion [1,4,5]. Because of these protective functions, the regulation of trehalose homeostasis is integrated into several key signalling pathways that govern growth and stress adaptation in yeast.

A major regulator of trehalose metabolism is the cAMP-protein kinase A (PKA) pathway, which modulates both TPS activity and Nth1-mediated trehalose degradation. Under favorable growth conditions, high PKA activity results in rapid Nth1 activation through multi-site phosphorylation, enabling trehalose mobilization to support biosynthesis and cell cycle progression [6,7]. Conversely, during stress or nutrient limitation, reduced PKA activity leads to Nth1 inactivation, stabilization of trehalose, and increased TPS function [2,8,9]. The transcription of *NTH1* is also responsive to environmental signals and is regulated by Msn2/Msn4 and other stress-responsive transcription factors [6.10]. Together, these mechanisms allow cells to rapidly switch between trehalose accumulation and degradation in a manner that aligns with metabolic demand, growth phase, and cellular stress status.

* Corresponding author: Tülay TURGUT GENÇ

The metabolic state of the cell further shapes trehalose regulation. During the post-diauxic shift (PDS), yeast transitions from fermentative to respiratory metabolism, accompanied by increased trehalose accumulation, activation of Snf1/AMPK, and global transcriptional reprogramming [11]. In this state, trehalose serves as both a carbon reserve and a molecular stabilizer supporting quiescence and long-term survival [12]. Crosstalk between PKA, Snf1, and TORC1 pathways ensures that trehalose turnover aligns with nutrient signalling and energy demand [8,13,14]. Disruption of these regulatory networks can cause profound defects in trehalose homeostasis and stress tolerance.

Boric acid is an essential micronutrient at low concentrations but becomes toxic at elevated levels, impairs growth and stress tolerance in yeast, plants, and other eukaryotes [15-17]. In *S. cerevisiae*, boron toxicity has been linked to oxidative stress, membrane damage, metabolic perturbation, and altered nutrient signalling [4,15,16]. However, despite its pronounced effects on cellular physiology, the impact of boric acid on trehalose metabolism remains poorly understood. Given the central role of PKA in trehalose turnover and the known sensitivity of PKA-dependent processes to stress signals, boric acid represents a compelling model for probing how environmental stress reshapes trehalose homeostasis across metabolic states.

In this study, we investigated how boric acid influences *NTH1* expression and trehalose accumulation during early growth phase, prolonged adaptation, and post-diauxic growth. By comparing wild type cells to a PKA-hyperactive mutant background, we aimed to dissect how signalling flexibility contributes to trehalose regulation under boron stress. Our findings reveal that boric acid exerts dynamic and phase-dependent control over trehalose metabolism, uncoupling *NTH1* transcription from trehalose turnover and highlighting the importance of metabolic context and PKA signalling in shaping cellular response to boron toxicity.

2. Materials and Methods

2.1. Yeast strains and transformation

The *Saccharomyces cerevisiae* strains *BY4741* (MATa, *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and *Σ1278b* (MATa, *ura3-52*) were used in this study [18,19]. The wild type strain, *BY4741*, does not harbour mutations affecting trehalose metabolism. In contrast, *Σ1278b* is characterized by intrinsically elevated intracellular cAMP levels and constitutively high PKA activity. Both yeast strains were obtained from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis).

NTH1 promoter activity was monitored using the pNL1 plasmid, which carries a transcriptional fusion of the *NTH1* promoter to the lacZ reporter gene. The construct contains 770 bp regulatory region upstream of the *NTH1* start codon, shown to encompass all cis-regulatory elements required for physiological *NTH1* expression. Yeast strains were transformed with pNL1 using the standard LA-PEG (lithium acetate-polyethylene glycol) method [20]. Transformants were selected on synthetic complete medium lacking uracil (Sc-Ura) supplemented with 2% glucose and incubated at 30 °C until single colonies developed. Single colonies were re-streaked onto fresh plates to ensure plasmid stability.

2.2. Growth conditions and boric acid treatment

Fresh transformants were used to inoculate SC-Ura medium supplemented with glucose, and cultures were incubated at 30°C with shaking until reaching mid-exponential phase (OD_{600}~ 0.5-0.6). Cells were harvested, washed and resuspended in fresh culture with or without boric acid. Boric acid was added to a final concentration of 50 mM Following medium renewal, cultures were incubated for either 90 min (early phase response) or 5 hr (late phase response) at 30°C. In parallel, experimental set-in which cells were not washed prior to boric acid treatment was prepared to allow adaptation under post-diauxic conditions, thereby distinguishing early phase responses from late-phase metabolic reprogramming. In this condition, boric acid was directly added to one of the continuous cultures and allowed to grow 5 hr at the same conditions.

2.3. Beta-galactosidase assay

Beta-galactosidase activity was used as a reporter for *NTH1* promoter activity and was quantified essentially as described previously [20]. Cells were harvested at the indicated time points, washed and resuspended in 200 µL breaking buffer. Samples were permeabilized by addition of 20 µL SDS (0.1%) and 20 µL chloroform. Beta-galactosidase was assayed using ONPG (o-nitrophenyl β-D-galactopyranoside) as substrate in Tris-HCl buffer. Protein concentrations were determined by the Lowry method [21]. Beta-galactosidase activity was expressed as Units indicating nmol of ONPG hydrolysed per minute per mg of protein (nmol ONPG/mg protein/min).

2.4. Trehalose quantification assay

Trehalose level in yeast cells were quantified following the principles of the method reported previously [22,23]. Briefly, approximately 60 mg wet cell mass was collected for each measurement and washed with ice-cold water. The cell pellet was then resuspended in 250 µL Na_2CO_3 (0.25 M) and boiled for 2 h to inactivate endogenous hydrolases and release reserve carbohydrates. After cooling, 150 µL acetic acid (1M) and 600 µL of 0.2 M sodium acetate buffer (pH 5.2) were added to adjust the sample to enzymatic reaction conditions. The resulting extract was used to trehalose determination. Trehalase (3mU; Sigma, T8778) was added to extracts and incubated at 37°C for 18 h for hydrolysing trehalose into glucose. The concentration of liberated glucose in reaction was measured using a glucose oxidase-peroxidase (GOD-POD) enzymatic colorimetric assay (Fluitest®- GLU, Biocon, Germany), according to the manufacturer's instructions. Trehalose content was expressed as a microgram of glucose equivalent per mg of wet cell mass (µg/mg).

2.5. Statistical analysis

All experiments were conducted with a minimum of three independent biological replicates per condition. Unless specified otherwise, each biochemical measurement was carried out in technical triplicates. To assess the combined influence of strain background, boric acid exposure, and recovery duration, levels of trehalose and glycogen, along with *NTH1* promoter activity, were detected, further analysis was performed using Tukey's post hoc test for multiple comparisons. Significance is reported as $p<0.05$ and results are expressed as mean ± standard deviation (SD).

3. Results

3.1. Early transcriptional modulation under boric acid exposure

During the early phase response (90 min), boric acid produced a markedly different *NTH1* promoter activity in the wild type compared with the PKA-hyperactive yeast cells (*Σ1278b*) (Figure 1). In the wild type, *NTH1* activity increased strongly under boric acid-free environment, reaching 3108 units, reflecting the characteristic early induction associated with metabolic reactivation. When boric acid was added, this activation was strongly suppressed, and promoter activity decreased to 1417 units, indicating that boric acid overrides the normal early-phase activation of *NTH1*.

In the PKA-hyperactive mutant, *NTH1* promoter activity remained uniformly low across all conditions. Under boric acid-free environment, the activity increased only slightly (613 units), highlighting the mutant's diminished capacity for transcriptional activation during early response. Under boric acid treatment, this activity declined even further to 162 units, indicating that boric acid enforces an even deeper transcriptional shutdown in a background already limited by chronically elevated PKA signalling.

Statistical analysis indicated a significant interaction among strain, treatment and time in the global three-way ANOVA ($F(8,28)=71.40$, $p<1.1\times10^{-15}$). This indicates that boric acid specifically blocks the strong early *NTH1* activation that normally occurs in the wild type, whereas the mutant strain which already unable to mount a strong induction due to hyperactive PKA signalling —undergoes an additional and pronounced repression.

3.2. Sustained transcriptional suppression during prolonged boric acid exposure

After long-term response (5h), boric acid imposed a persistent repression of *NTH1* promoter activity (Figure 2). In the wild type, promoter activity stabilized around 500 units under boric acid-free conditions, defining the long-term steady-state level after initial response. The presence of boric acid reduced this activity to 214 units, indicating a sustained inhibitory effect that keeps *NTH1* expression well below the untreated steady state. The mutant strain showed an even more restricted profile. In the absence of boric acid, *NTH1* activity remained low at 75 units, and boric acid further decreased this value to 37 units. Thus, prolonged exposure compresses promoter activity to the lower limit expression in the mutant, which is already unable to achieve the wild type of transcriptional level under boric acid presence. Throughout long-term response, the mutant remains uniformly low-expressing across all conditions, whereas the wild type retained a higher expression plateau that is specifically driven down by boric acid. Taken together, these findings indicate that prolonged boric acid exposure enforces a deep and sustained shutdown of *NTH1* expression in the wild type, while the mutant strain—already transcriptionally constrained—remains locked in a uniformly low-expression state that becomes further reduced under treatment.

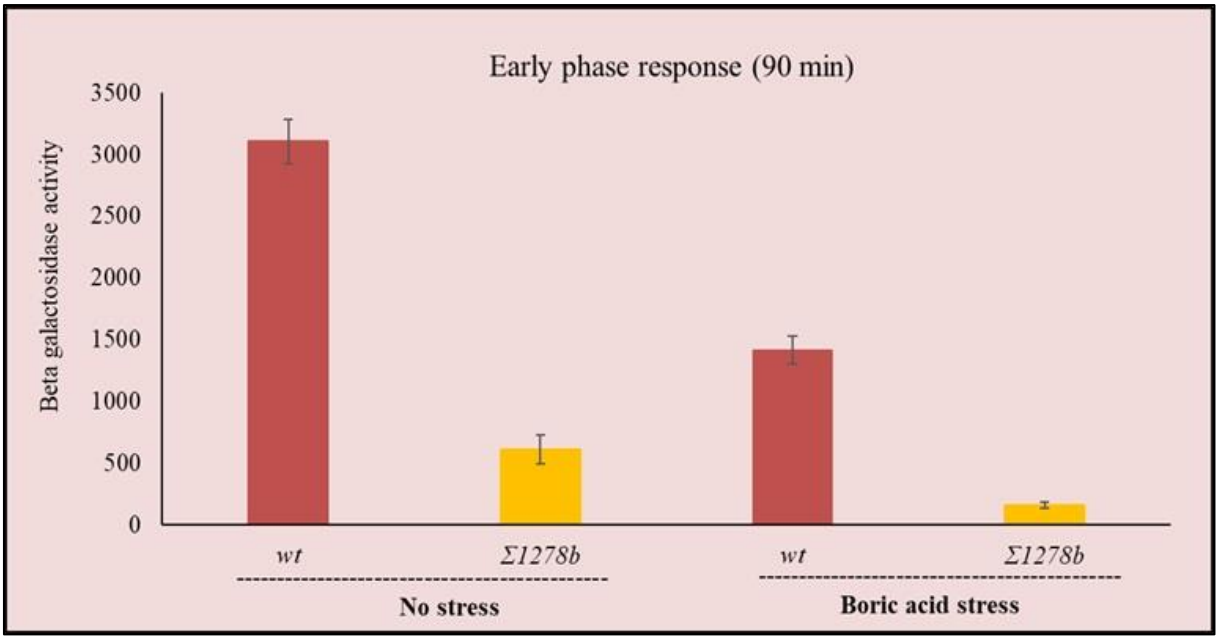

Figure 1 Immediate transcriptional response of *NTH1* to boric acid.

3.3. Post-diauxic shift alters the direction of boric acid-mediated NTH1 transcription

In post-diauxic cultures, boric acid elicited a regulatory pattern that differed from that observed during exponential phase response (Figure 3). In the wild type, *NTH1* promoter activity was 156 units under boric acid-free conditions but increased to 214 units in the presence of boric acid, revealing a clear activation instead of repression. This shift indicates that the metabolic state of the cells determines the direction of the *NTH1* response: a treatment that suppresses *NTH1* during exponential phase response becomes a modest activating signal following the diauxic transition. In the mutant strain, however, this metabolic-state-dependent reversal was absent. *NTH1* activity remained very low (31 units) under boric acid-free environment and did not show any meaningful induction upon boric acid treatment (23 units). The mutant therefore fails to execute the transcriptional switch observed in the wild type and remains locked in a low expression state regardless of metabolic phase or treatment. Together, these results show that boric acid reverses its regulatory impact on *NTH1* specifically in post-diauxic wild type cells, whereas the mutant strains lack this adaptive flexibility and remains transcriptionally unresponsive.

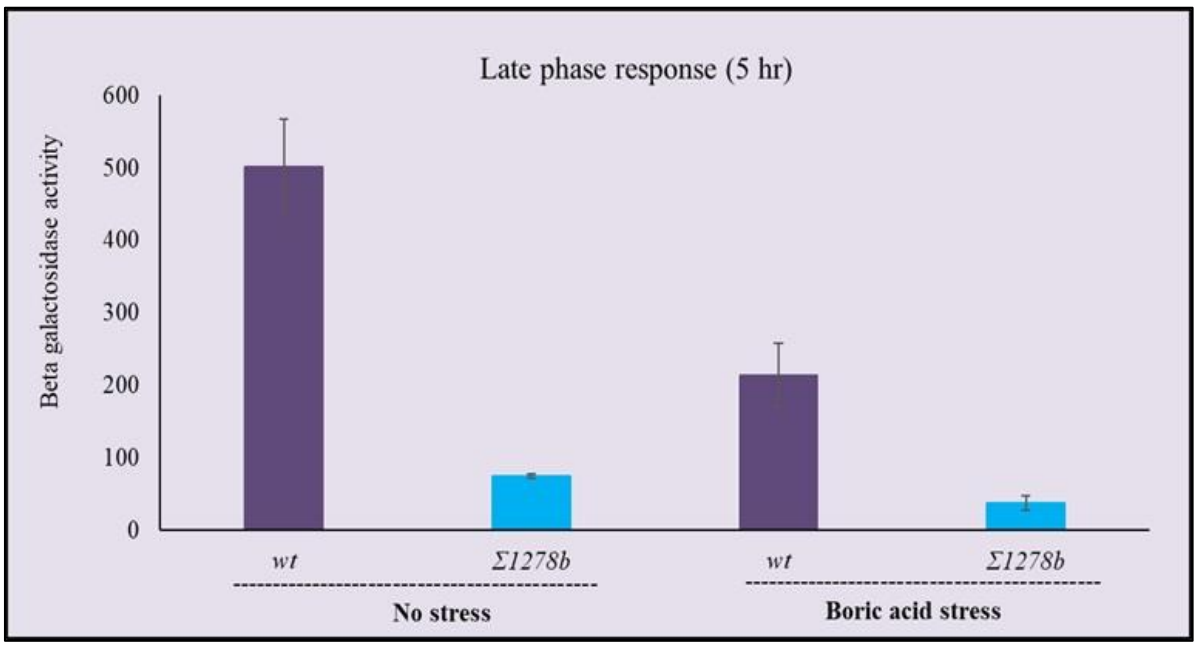

Figure 2 Extended-phase response of *NTH1* expression to boric acid.

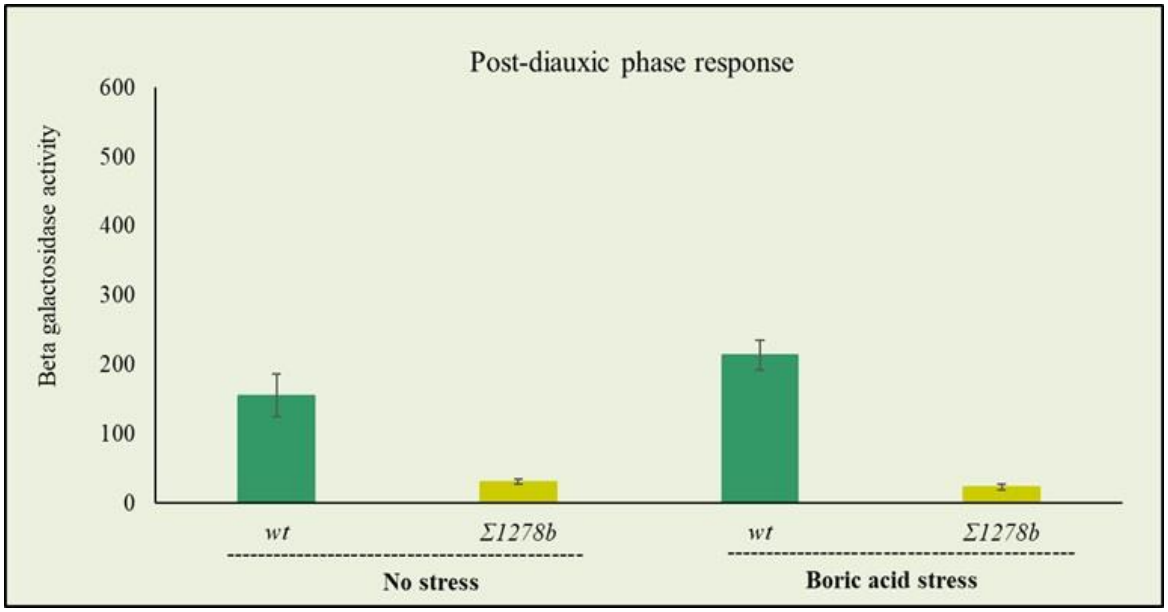

Figure 3 Post-diauxic transcriptional behaviour of *NTH1* in response to boric acid.

3.4. Trehalose accumulation under boric acid exposure

Trehalose quantification revealed distinct and phase-dependent regulatory effects of boric acid in the two yeast background (Figure 4). During early phase response, boric acid markedly enhanced trehalose accumulation in the wild type, increasing levels from 766 to 1914 units. The mutant strain also showed an increase under boric acid treatment, although to a lesser extent (from 197 to 667 units), indicating a limited but detectable induction capacity. These patterns demonstrate that boric acid functions as a potent early stimulus for trehalose synthesis, with the wild type exhibiting a considerably stronger metabolic activation.

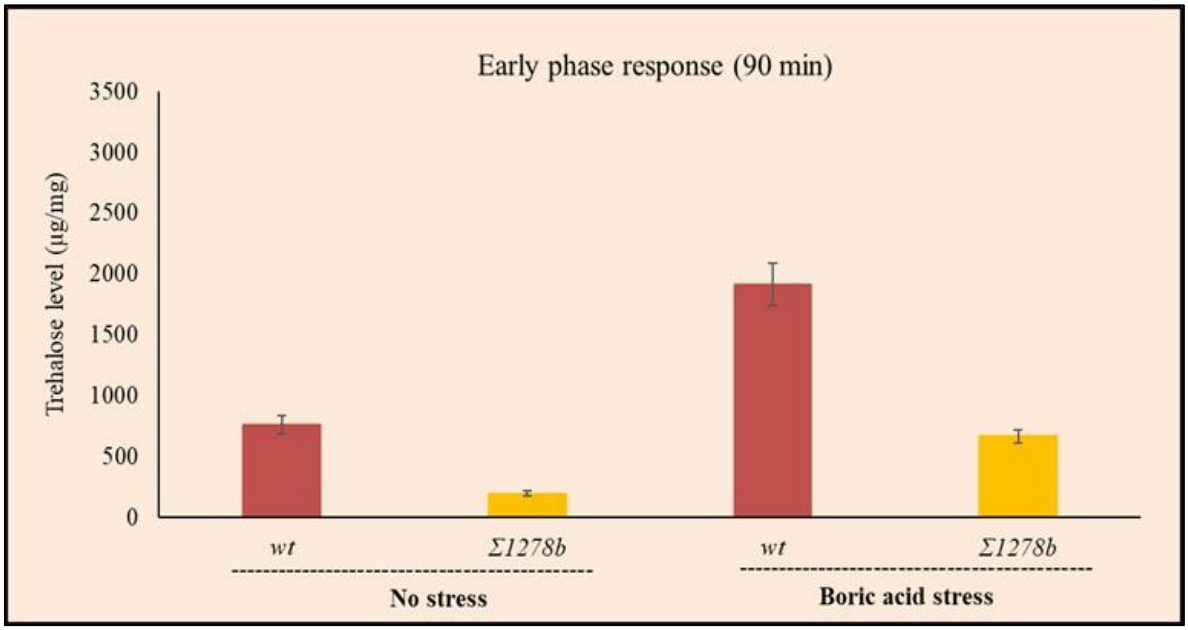

Figure 4 Early phase induction of trehalose accumulation under boric acid exposure.

A different regulatory profile emerged during long-term response (Figure 5). In the wild type, trehalose reached its highest levels under untreated conditions (1246 units) but dropped sharply to 423 units in response to boric acid, suggesting that prolonged exposure suppresses trehalose synthesis after the initial adaptive phase. The mutant, in contrast, exhibited a more compressed dynamic range; trehalose remained at 558 units in untreated cultures and

decreased only modestly under boric acid treatment. This indicates that the mutant strain lacks both the strong early induction and the pronounced long-term repression observed in the wild type.

In post-diauxic cultures, where respiratory metabolism predominates, trehalose levels increased substantially in both strains. Boric acid reduced trehalose accumulation from 2467 to 1050 units in the wild type, demonstrating that the suppressive effect of boric acid persists beyond exponential growth. The mutant strain, however, maintained consistently high trehalose levels (2768 *vs* 2391 units), displaying only minimal reduction. Thus, the mutant remains largely refractory to boric acid-mediated suppression under post-diauxic conditions.

Statistical analysis confirmed strong effects of boric acid on trehalose dynamics, as indicated by highly significant strain × condition × time interaction ($F(8,28)=28.73$, $p<1.63 \times 10^{-10}$). Together, these results show that boric acid enhances trehalose synthesis only during early phase, but suppresses it during prolonged and post-diauxic phases, with the magnitude and direction of the response strongly dependent on strain background and metabolic state.

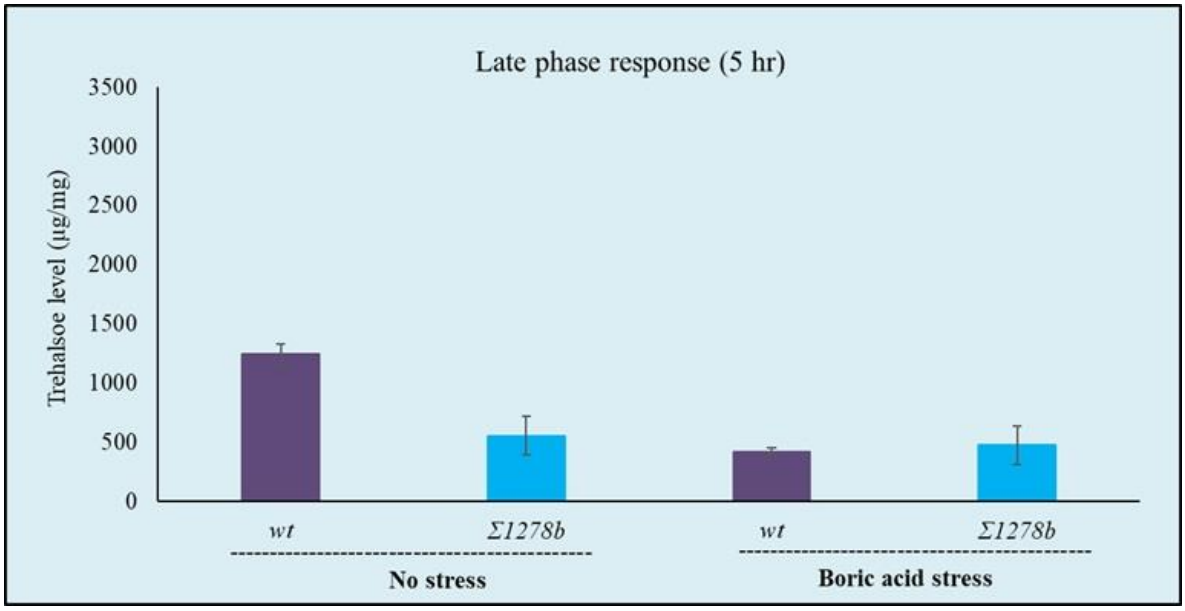

Figure 5 Long-term suppression of trehalose levels during prolonged boric acid exposure.

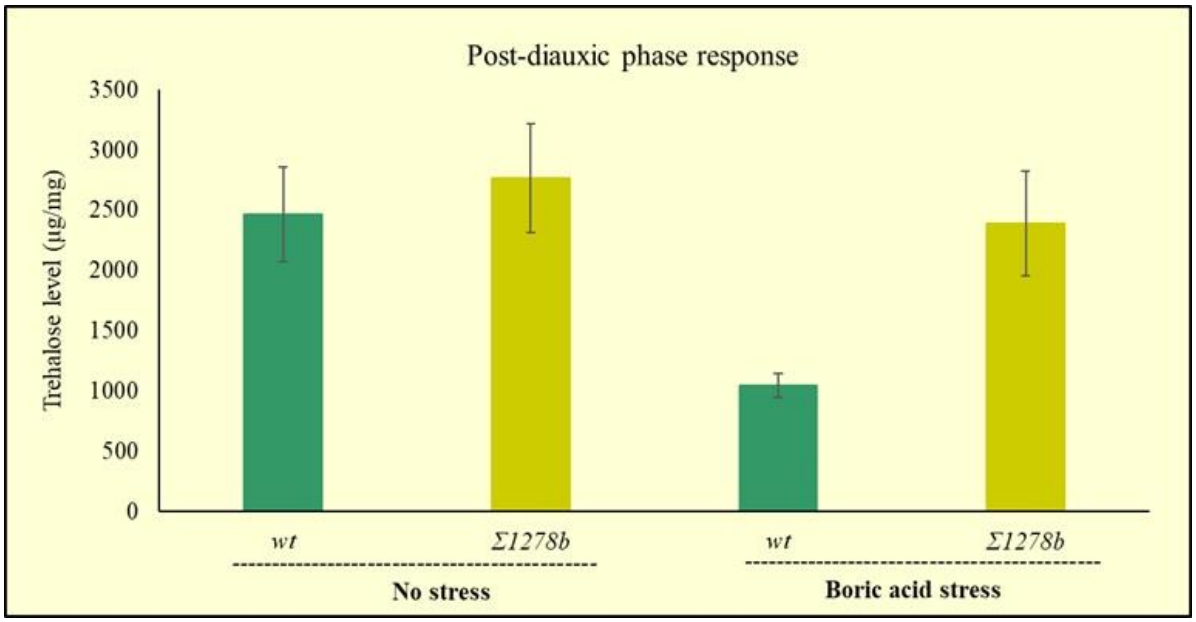

Figure 6 Trehalose accumulation during the post-diauxic phase under boric acid exposure

4. Discussion

This study reveals a complex, phase-dependent regulation of trehalose metabolism under boric acid-induced stress, characterized by a striking divergence between *NTH1* promoter activity and trehalose accumulation. During the early phase response, boric acid suppressed *NTH1* promoter activity yet strongly stimulated trehalose synthesis. This apparent discrepancy is consistent with the known regulatory principles of trehalose metabolism, although *NTH1* transcription can be stress responsive, Nth1 enzyme activity strictly requires PKA-dependent multisite phosphorylation at Ser60, Ser83, and Ser20, and therefore remains inactive when PKA signalling is inhibited [6,7]. Stress conditions such as nutrient limitation, or osmotic/thermal shock rapidly activate the TPS complex, driving trehalose accumulation, while trehalase remains inactive, allowing trehalose to function as a protective osmolyte and protein stabilizer [2,4,15,24-26]. Our findings align closely with this framework, suggesting that boric acid acts simultaneously as a metabolic stressor that stimulates trehalose synthesis via TPS complex activation and prevents trehalose degradation by repressing *NTH1* expression and PKA-dependent activation, thereby favouring trehalose preservation.

The marked early accumulation of trehalose in the wild type compared to the mutant further supports the requirement for tightly regulated PKA activity in trehalose regulation. Elevated PKA signalling is known to inhibit TPS activation and constitutively repress stress-responsive transcription factors such as Msn2/Msn4 [1,4,27]. The *Σ1278b*-derived PKA-hyperactive mutant examined here displayed weak early trehalose induction even under boric acid stress, mirroring previously reported phenotypes in hyper-PKA backgrounds that show attenuated stress tolerance and defective trehalose accumulation [22,28].

During long-term response, trehalose levels decreased in boric acid-treated wild type cells while *NTH1* promoter activity remained repressed. This suggests extended boron exposure disrupts trehalose homeostasis through impaired synthesis or altered metabolic allocation rather than accelerated degradation. Long-term stress is known to modulate TORC1-Sch9 and Snf1 pathways, which regulate reserve carbohydrate turnover and metabolic remodeling during adaptation [29-31]. Boron toxicity has been associated with mitochondrial dysfunction, oxidative stress, and shifts in carbon metabolism, all of which may limit trehalose biosynthesis under prolonged exposure [4,15,16]. The compressed trehalose dynamics of the mutant —characterized by attenuated early induction and mild long-term suppression— further support the necessity of flexible PKA regulation for proper trehalose cycling during stress.

Post-diauxic shift (PDS) conditions revealed an additional regulatory configuration. Trehalose naturally accumulates during respiratory metabolism, driven by Snf1 activation and global transcriptional restructuring [11,31]. Here, boric acid markedly reduced trehalose in the wild type while modestly inducing *NTH1* promoter activity, opposite to the early recovery response pattern. This metabolic state-dependent reversal is consistent with evidence that trehalose mobilization in PDS can be mediated not only by PKA but also through alternative pathways such as Pho85-Pho80, Snf1, and mitochondrial pathways [9,14]. Importantly, boron's capacity to modulate mitochondrial function and induce reactive oxygen species (ROS) —documented in both plant and yeast models— may further alter trehalose turnover in PDS cells by reshaping carbon partitioning and stress responses [15,16,32]. The mutant strain, however, showed little response, consistent with the idea that constitutive PKA activity disrupts its ability to reprogram carbohydrate metabolism across different metabolic states.

Previous studies have shown that the PKA pathway is not required for the global boron stress response [32]. Our data refine this view by showing that, although PKA is dispensable for the core boron tolerance program, its regulatory state influences the trehalose branch of the response. The PKA-hyperactive mutant fails to accumulate trehalose during the early phase of boron stress, suggesting that chronic PKA activity limits the magnitude of the trehalose surge observed in the wild type cells but does not serve as it initiating signal. Because trehalose synthesis in the wild type is still activated when *NTH1* is repressed, pathways upstream of PKA are likely responsible for triggering this response. Although TOR activity was not directly assessed in our study, previous work has shown that boron toxicity interferes with TORC1 signalling, raising the possibility that TOR-dependent mechanisms, rather than PKA, primarily drive trehalose remodeling under boron stress, with PKA dysfunction becoming evident only under hyperactive conditions.

Collectively, these findings support a model in which boric acid disrupts the reciprocal regulation of trehalose synthesis and degradation by modulating transcriptional control of *NTH1*, altering PKA-dependent enzyme activation, and reshaping carbon flux in a metabolic state-dependent manner. The significant strain × condition × time interactions indicate that trehalose homeostasis operates through multilayered regulatory circuits that are highly sensitive to genetic background and metabolic context. These insights expand understanding of how boron stress perturbs energy storage pathways and emphasize the central role of PKA-mediated flexibility in metabolic adaptation.

5. Conclusion

This study shows that boric acid reshapes trehalose metabolism in a highly dynamic and phase-dependent manner, suppressing *NTH1* transcription while modulating trehalose accumulation according to metabolic state and genetic background. The wild type displayed a flexible trehalose cycle, characterized by strong early induction, pronounced long-term suppression and a metabolic-state-dependent reversal after the diauxic shift, whereas the PKA-hyperactive strain remained transcriptionally and metabolically constrained throughout. These findings reveal that boric acid disrupts the balance between trehalose synthesis and degradation by interfering with *NTH1* regulation and signalling pathways that coordinate carbohydrate homeostasis. Overall, the work provides a mechanistic framework for understanding how boron stress affects reserve carbohydrate dynamics in yeast and highlights the importance of PKA-mediated flexibility in maintaining metabolic resilience.

Compliance with ethical standards

Disclosure of conflict of interest

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

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