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## Roles of *ATR1* paralogs *YMR279c* and *YOR378w* in boron stress tolerance

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### ABSTRACT

Boron is a necessary nutrient for plants and animals, however excess of it causes toxicity. Previously, *Atr1* and *Arabidopsis* *Bor1* homolog were identified as the boron efflux pump in yeast, which lower the cytosolic boron concentration and help cells to survive in the presence of toxic amount of boron. In this study, we analyzed *ATR1* paralogs, *YMR279c* and *YOR378w*, to understand whether they participate in boron stress tolerance in yeast. Even though these genes share homology with *ATR1*, neither their deletion rendered cells boron sensitive nor their expression was significantly upregulated by boron treatment. However, expression of *YMR279c*, but not *YOR378w*, from the constitutive *GAPDH* promoter on a high copy plasmid provided remarkable boron resistance by decreasing intracellular boron levels. Thus our results suggest the presence of a third boron exporter, *YMR279c*, which functions similar to *ATR1* and provides boron resistance in yeast.

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### 1. Introduction

Boron is an essential micronutrient for various species [1–4]. The essentiality of boron is known almost for 90 years for plants [5]. In plants, boron act as a cross-linker of rhamnogalacturonan-II molecule [6] within the cell wall and maintains stability of the plant cell structure, and its deficiency has been linked to growth problems in *Arabidopsis thaliana* [4].

*A. thaliana* *Bor1* is the first identified boron transporter, functions as an efflux-pump and essential when limited amount of boron is available for growth [7]. Yeast *Bor1* has been identified as a boron exporter having role in boron tolerance [8]. In a recent study, *Atr1* was suggested as the main boron exporter in yeast [9]. *Atr1* deficient cells were boron sensitive and accumulated higher level of intracellular boron, whereas *ATR1* overexpressing cells were resistant to toxic amount of boron application and contained less boron. *ATR1* transcription was induced several fold by boron treatment [9].

Yeast *ATR1* belongs to the DHA2 family of drug: H<sup>+</sup> antiporters with 10 members in the budding yeast, and this transporter family is conserved among yeast species such as *Kluyveromyces lactis*, *Kluyveromyces waltii*, *Candida glabrata*, *Ashbya gossypii* [10]. Seven of DHA2 family members including *YMR279c* and *YOR378w* have not yet been characterized [11]. Since *YMR279c* and *YOR378w* are the two closest paralogs of *ATR1*, we speculated that they may play a role in boron stress response. To test this idea, both genes were cloned and overexpressed in yeast. Expression of

*YMR279c*, but not *YOR378w*, provided boron resistance by decreasing intracellular boron level.

### 2. Materials and methods

#### 2.1. Yeast growth and media

WT strain BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic *atr1Δ*, *ymr279cΔ*, and *yor378wΔ* deletion mutants were obtained from the yeast deletion library (Invitrogen). YPD (2% glucose, 2% peptone, 1% yeast extract, and 2% agar for solid media) and YNB media (0.67% yeast nitrogen base without amino acids, 2% glucose, 2% agar for solid media) were used for cell growth. For spotting assays, overnight cultures were diluted to an optical density of 0.2, 0.02, 0.002, and 0.0002 at 600 nm by serial dilution. Five microliters of each dilution was spotted on YNB-URA agar plates which contain different amounts of boric acid. Plates were incubated at 30 °C for 3 days and then photographed. For growth curves, overnight cultures were diluted to an optical density of 0.05 for YPD at 600 nm. The cultures were incubated in YPD media containing 50 mM boric acid and OD<sub>600</sub> measurements were taken every 2 h.

#### 2.2. Cloning of the *YMR279c* and *YOR378w* genes

The cloning of the *YMR279c* and *YOR378w* genes were achieved by using the Gateway Cloning System (Invitrogen). Wild type alleles of *YMR279c* and *YOR378w* genes were amplified by Long PCR Enzyme Mix (Fermentas) from yeast genomic DNA. The amplified fragments were first cloned into pDONR vector and then moved into pAG426GPD. Plasmids were confirmed with sequence

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analyses. Standard lithium acetate procedure was used for yeast transformation.

### 2.3. Determination of intracellular boron concentrations

Wild type and *ymr279cΔ* cells were grown to logarithmic phase and then splitted into two cultures. One group was treated with 50 mM boric acid whereas the other group remained untreated. After 1 h of incubation at 30 °C, cells were harvested and washed with distilled water three times. Cell pellets were disrupted in 500 μl lysis buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA pH 8, 2% Triton X-100, 1% sodium dodecyl sulfate, 10 mg/ml protease inhibitor cocktail [Sigma], 500 μl glass beads) by vortexing. The intracellular boron concentrations were determined by Boron Cell Test kit (Merck) using cell extracts. Samples were normalized by cell numbers. The measurement sensitivity of the kit was  $8 \times 10^{-7}$  M (0.05 mg boron/L) as stated by the manufacturer.

### 2.4. RNA isolation and real-time PCR analyses

Total RNA isolations from 50 mM boric acid treated or untreated samples were performed using the PureLink RNA kit (Invitrogen) and genomic DNA contaminations were removed by DNase treatment (Fermentas). For cDNA synthesis, the First Strand cDNA synthesis kit (Fermentas) was used. *ATR1*, *YMR279C* and *YOR378W* genes were amplified by using Fermentas Maxima™ SYBER Green qPCR Master Mix (2×). *ACT1* gene was used as the internal control. Real-time PCR experiments were performed with an IQ5 real-time PCR system (Bio-Rad) and conditions were as follow: 40 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The primers used for *ATR1* amplification were *ATRRT1F* (5'-GGCAGAC TAGGTGACATATACG-3') and *ATRRT1R* (5'-TTGTTTTGGGTCCTCGGT AC-3'). The primers for *YMR279c* amplification were *YMRRTF* (5'-TTTCGATGGATTGGACTGGG-3') and *YMRRTR* (5'-TCCAAATACAAA CGACCC-3') and those used for *YOR379w* were *YORRTF* (5'-ATGGGCTTACTGGATAATGGG-3) and *YORRTR* (5'-AATGGTAGCA-GAGGGAAAGC-3'). Finally, the primers used for *ACT1* gene amplification were *ACT1F* (5'-ACGTTCCAGCCTTCTACGTTTCCA-3') and *ACT1B* (5'-ACGTGAGTAACACCATCACCGGAA-3').

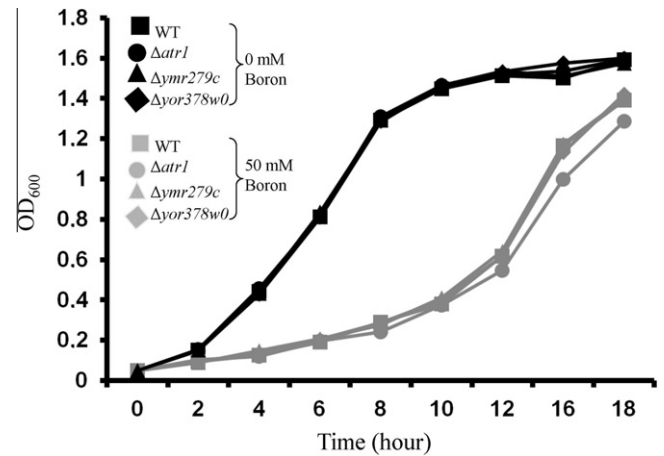
### 2.5. Bioinformatic analyses

Multiple sequence alignments were performed via T-coffee 6.85 [12] software using the default parameters. Pairwise sequence alignments were performed with the Lalign 2.1.30 on-line version, and sequence identity percentages were calculated using the same software on DNA scoring matrix via default parameters [13].

## 3. Results

### 3.1. Boron effects on cell growth

Cells lacking *YMR279c* and *YOR378w* genes were previously tested for boron sensitivity and they were found to be not boron sensitive [9]. Based on this observation, both *YMR279c* and *YOR378w* genes have not been considered for further investigation



**Fig. 1.** Boric acid tolerance levels of *atr1Δ*, *ymr279cΔ* and *yor378wΔ* mutants. Overnight cultures of wild type, *atr1Δ*, *ymr279cΔ* and *yor378wΔ* cells were diluted to an optical density of 0.05 and incubated in the presence or absence of 50 mM boric acid. Cell growth was followed by OD<sub>600</sub> measurements.

for their roles in boron tolerance. However, when their primary structures are considered, *Ymr279c* and *Yor378w* show 65% and 40% homology to *Atr1*, respectively. The N-terminal region is the most heterogeneous part in all three proteins (Fig. S1). In spite of the similarities in their ORFs, they do not contain similar consensus sequences and are not regulated by common transcription factors (Table 1) as deduced from the YeastRACT (Yeast Search for Transcriptional Regulators and Consensus Tracking) database [14]. There is only one transcription factor, Cin5, seems to regulate both *ATR1* and *YMR279c*. Cin5 is a basic leucine zipper transcription factor that belongs to the Yap1 family and plays a role in multidrug resistance [15,16]. On the other hand, Met4 is the only transcription factor which is common to both *ATR1* and *YOR378w* genes. Met4 plays a role in biosynthesis of sulfur containing amino acids [17]. Regulation of *ATR1* and its paralogs by different transcription factors suggest that they may function under different stress conditions.

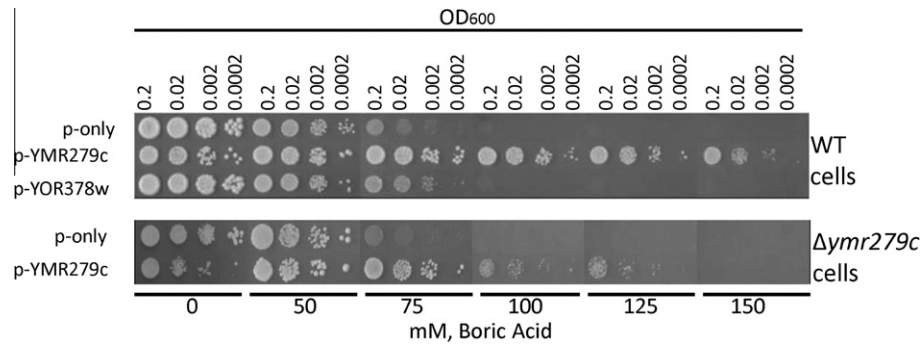
The lack of regulatory link between *ATR1* and its paralogs prompted us to further analyze them regarding their roles in boron stress tolerance. Specifically, we wanted to know whether transcriptional regulation of *YMR279c* and *YOR378w* or sequence differences in their ORFs is responsible for boron insensitive phenotypes of *ymr279cΔ* and *yor378wΔ* mutants as noticed previously [9]. We first confirmed the results of Kaya et al. [9] by checking the growth rates of the mutants on boron containing media. Both *ymr279cΔ* and *yor378wΔ* mutants could grow well in media containing 50 mM of boron, whereas *atr1Δ* cells grew slightly slower (Fig. 1). Thus, absence of *YMR279c* and *YOR378w* genes does not sensitize cells for boron. In order to see whether overproduction of these genes from a constitutive promoter on a high copy number plasmid would help to tolerate boron, both genes were cloned and overexpressed in wild-type cells. As seen in Fig. 2 (upper panel), overexpression of *YMR279c*, but not *YOR378w* provided a significant level of boron tolerance to

**Table 1**

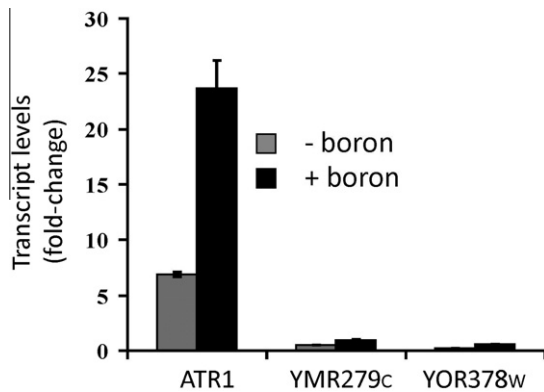
Transcription factors shown to regulate *ATR1* and its paralogs.

Gene name	Transcription factors
<i>ATR1</i>	Cad1p, Cin5p, Fhl1p, Fkh2p, Gcn4p, Met4p, Msn2p, Msn4p, Pdr1p, Pdr3p, Phd1p, Put3p, Rim101p, Rpn4p, Sfp1p, Stp2p, Yap1p, Yap6p, Yap7p
<i>YMR279C</i>	Aft1p, Aft2p, Cat8p, Cin5p, Mal33p, Mbp1p, Rfx1p, Swi4p
<i>YOR378W</i>	Adr1p, Crz1p, Gal4p, Met4p, Rap1p, Rdr1p, Rfx1p, Sok2p, Ste12p, Swi4p

Data were extracted from YEASTRACT database (14), using the search option for transcription factors.



**Fig. 2.** Overexpression of *YMR279C* confers boric acid resistance to wild type and *ymr279cΔ* cells. Wild type cells were transformed with an empty vector (p only) or p-YMR279C or p-YOR378W plasmids. The transformants were grown to logarithmic phase in YNB-Ura medium, diluted to an OD of 0.2, 0.02, 0.002, and 0.0002 at 600 nm, and 5  $\mu$ l of each cell suspension was spotted on YNB-Ura medium containing indicated amounts of boric acid. The plates were incubated at 30 °C for 3 days and photographed (upper panel). The *ymr279cΔ* mutant cells were transformed with an empty vector or pAG426GPD-YMR279C plasmid and the transformants were spotted on YNB-Ura plates containing indicated amounts of boric acid and incubated at 30 °C for 3 days (lower panel).

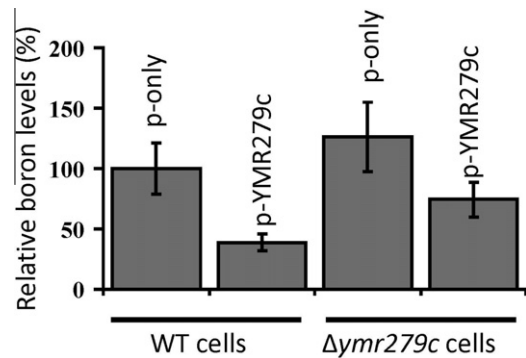


**Fig. 3.** Transcriptional expression profiles of *YMR279C* and *YOR378W* in response to boric acid treatment. Wild type cells were grown to logarithmic phase and were split into two groups, one of them was treated with 50 mM boric acid for 1 h. *ATR1*, *YMR279C* and *YOR378W* mRNA levels were detected by a real-time PCR analyses. *ACT1* was used as the internal control.

wild-type cells. *YMR279c* overexpression also provided boron resistance to *ymr279cΔ* mutants (Fig. 2, lower panel). Thus, constitutive high level expression of *YMR279c* helps cells to tolerate higher concentration of boron.

### 3.2. Expression analyses of *YMR279c* and *YOR378w* in response to boron

A physiological stress response gene is expected to be induced upon treatment with its stressor. Since *YMR279c* can function as a boron tolerance gene, we investigated whether boron stress is transduced to transcriptional machinery responsible for *YMR279c* transcription. To do so, cells were challenged with boron and the transcript levels of *ATR1*, *YMR279c*, and *YOR378w* genes were analyzed in parallel by a real-time PCR approach. As seen in Fig. 3 basal levels of *YMR279c* and *YOR378w* transcripts were 14-fold and 34-fold lower, respectively, in untreated cells when compared to that of *ATR1*, and they showed a less than 2-fold increase upon boron treatment. However, high basal level of *ATR1* mRNA was further upregulated by 4-fold in response to boron. This mode of *ATR1* regulation was previously noticed [9]. Thus, even though *Ymr279c* has a role in boron detoxification, its transcription is not significantly regulated by boron stress, which suggest that *Ymr279c* role in boron tolerance may not be very important under native conditions.



**Fig. 4.** Intracellular boron levels in wild-type and *ymr279cΔ* cells that were exposed to 50 mM boric acid for 1 h. Cells were transformed with either empty vector or *YMR279C* overexpression plasmid (p-YMR279C). Boron levels were normalized by total cell counts. Error bars represent three separate measurements.

### 3.3. *YMR279c* expression lowers intracellular boron level after boron treatment

Previously two membrane proteins, Bor1 and Atr1, were shown to provide boron resistance by reducing intracellular boron levels [8,9]. In order to test whether *Ymr279c* functions as boron efflux transporter similar to Bor1 or Atr1, intracellular boron levels of cells overexpressing *YMR279c* or containing sham vector were determined after boron application. As shown in Fig. 4, *ymr279cΔ* cells with empty plasmid had slightly higher (25%) amount of boron when compared to that of empty plasmid carrying wild-type cells. *YMR279c* overexpression in both wild-type and *ymr279cΔ* mutants decreased cellular boron levels by 60% and 40%, respectively, when compared to their corresponding sham vector carrying cells. These results suggest that *YMR279c* expression help cells to lower intracellular boron concentration when they are exposed to boron stress.

## 4. Discussion

In yeast, mechanisms of boron resistance are centered around exportation of boron out of cells. Two membrane proteins, Atr1 and Bor1, were previously characterized as membrane boron efflux transporters [9,18]. Here we showed that *Ymr279c*, one of the two paralogs of Atr1, provides boron resistance by decreasing intracellular boron concentrations similar to Atr1. However, there are differences between Atr1 and *Ymr279c* roles in boron tolerance.

*YMR279c* is slightly upregulated by boron at the transcriptional level and provides boron resistance only if it is expressed ectopically from a constitutive promoter. Moreover, its overexpression leads to less boron resistance when compared to *ATR1*, whose expression leads to toleration of up to 220 mM boric acid [9].

Interestingly, when Kaya et al. [9] screened a yeast gDNA library for the genes that confer resistance for boron in wild-type cells, *YMR279c* gene was not identified as a boron tolerance gene. A possible explanation for this outcome is that *YMR279c* containing cells were probably not able to grow under the experimental conditions, because they picked the transformants that could tolerate up to 200 mM of boron. Here we showed that *YMR279c* overexpression helps to tolerate only about 150 mM of boron. In a separate genome-wide screening, yeast deletion mutants that are sensitive or resistant to boron stress were identified, but *ymr279cΔ* mutants escaped from detection [19], because of the fact that *ymr279cΔ* cells are not boron sensitive. Thus, our special interest in testing *ATR1* paralogs for boron stress response resulted in identification of *YMR279c* as the third boron efflux transporter in yeast.

Gene families are usually formed via gene duplication events and additional copies of same genes confer selective advantages to the host [20] by undergoing an accelerated evolution [21]. Thus, members of gene families may obtain novel functions and gain different regulatory patterns. It seems that *Atr1* and its paralogs have been differentiated both function- and regulation-wise over the course of the evolution. Among them, *Atr1* is the main boron exporter, *Ymr279c* has potential to pump boron out of cell and *Yor379w* has no roles in boron tolerance.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.080.

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