

Identification of a Novel System for Boron Transport: Atr1 Is a Main Boron Exporter in Yeast^{∇†}

Alaattin Kaya,^{1,2} Huseyin C. Karakaya,¹ Dmitri E. Fomenko,²
Vadim N. Gladyshev,² and Ahmet Koc,^{1*}

Department of Molecular Biology and Genetics, Izmir Institute of Technology, 35430 Urla, Izmir, Turkey,¹ and Department of Biochemistry and Redox Biology Center, University of Nebraska, Lincoln, Nebraska 68588²

Received 22 October 2008/Returned for modification 20 January 2009/Accepted 20 April 2009

Boron is a micronutrient in plants and animals, but its specific roles in cellular processes are not known. To understand boron transport and functions, we screened a yeast genomic DNA library for genes that confer resistance to the element in *Saccharomyces cerevisiae*. Thirty boron-resistant transformants were isolated, and they all contained the *ATRI* (YML116w) gene. Atr1 is a multidrug resistance transport protein belonging to the major facilitator superfamily. C-terminal green fluorescent protein-tagged Atr1 localized to the cell membrane and vacuole, and *ATRI* gene expression was upregulated by boron and several stress conditions. We found that *atr1Δ* mutants were highly sensitive to boron treatment, whereas cells overexpressing *ATRI* were boron resistant. In addition, *atr1Δ* cells accumulated boron, whereas *ATRI*-overexpressing cells had low intracellular levels of the element. Furthermore, *atr1Δ* cells showed stronger boron-dependent phenotypes than mutants deficient in genes previously reported to be implicated in boron metabolism. *ATRI* is widely distributed in bacteria, archaea, and lower eukaryotes. Our data suggest that Atr1 functions as a boron efflux pump and is required for boron tolerance.

Boron has been proposed as an important micronutrient in plants and animals. Studies have shown the presence of several genes associated with boron transport and tolerance in plants (18, 25, 27); however, boron transport mechanisms in other organisms, including animals, remain unclear. In plants, boron functions as a cross-linker for rhammoglacturonan II in the cell membrane (9, 14, 21) and also as a structural component in cytoskeleton assembly (1). *Arabidopsis thaliana* *BOR1* was the first gene shown to play a role in boron tolerance (28). Homologs of *BOR1* were found in many organisms, including yeasts, plants, and mammals (22, 25, 29). A high level of boron leads to degradation of its own exporter, *BOR1*, in *A. thaliana* (27), and *A. thaliana* *BOR1* cannot be used to produce genetically modified plants that grow in soil with high boron levels. However, transgenic plants expressing *BOR4*, one of six paralogs of *BOR1*, showed high tolerance to toxic levels of boron (18). Multicopy expression of *BOT1*, a *BOR1* ortholog, provided boron tolerance to barley (25).

The yeast *Saccharomyces cerevisiae* has been used as a model organism for characterization of plant boron tolerance genes (19, 20, 25, 26, 29). While 10 mM boric acid is lethal to *Arabidopsis* (18), yeast can grow in the presence of 80 mM boron and is considered a boron-tolerant organism (19, 20). Yeast Bor1 was characterized in detail (10). This protein is localized to the plasma membrane and functions as a boric acid exporter (26). The *bor1Δ* yeast strain overaccumulates boron (20, 28), and cells that overexpress *BOR1* have less intracellular boron and show

resistance to boron treatment (20). In addition to Bor1, two other proteins, Dur3 and Fps1, have been implicated in boron tolerance in yeast, but their functions are not clear (20). Dur3 is a plasma membrane transporter that plays a role in urea and polyamine transport (5, 31), and Fps1 is a member of the major intrinsic protein family and plays a role in glycerol, acetic acid, arsenite, and antimonite transport (16, 30, 33). Overexpression of *FPS1* and *DUR3* showed controversial effects on cellular boron levels. While *FPS1* expression lowered the protoplasmic boron concentration, *DUR3* expression led to a small increase in boron (20).

The objective of this study was to identify proteins that are primarily responsible for boron transport in yeast. *ATRI* was identified as a boron tolerance gene by screening a yeast DNA expression library. Yeast Atr1 is a member of the DHA2 family of drug-H⁺ antiporters with 14 predicted membrane-spanning segments (7). It was first characterized in a genetic screen as a high-copy-number suppressor of the 3-amino-1,2,4-triazole sensitivity of *gcn4Δ* mutants (11). It also conferred resistance to the DNA-damaging agent 4-nitroquinoline-*N*-oxide in a separate genetic screen (17). In this study, we demonstrated that high-copy-number expression of *ATRI* conferred extreme resistance to boron and reduced intracellular levels of the element, whereas cells lacking the *ATRI* gene were hypersensitive to boron and increased its intracellular levels. We analyzed changes in the global gene expression profile in response to boron and found that *ATRI* is the most induced transporter gene. The Atr1-green fluorescent protein (GFP) fusion protein localized to the plasma membrane and vacuole. Taken together, our data show that Atr1 functions as a major boron efflux pump and provides tolerance of the element by pumping boron out of cells.

* Corresponding author. Mailing address: Department of Molecular Biology and Genetics, Izmir Institute of Technology, 35430 Urla, Izmir, Turkey. Phone: 90-232-750 7544. Fax: 90-232-750 7509. E-mail: ahmetkoc@iyte.edu.tr.

† Supplemental material for this article may be found at <http://mcb.asm.org/>.

∇ Published ahead of print on 4 May 2009.

MATERIALS AND METHODS

Yeast strains. The wild-type strain BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic deletion mutants were obtained from the yeast deletion library

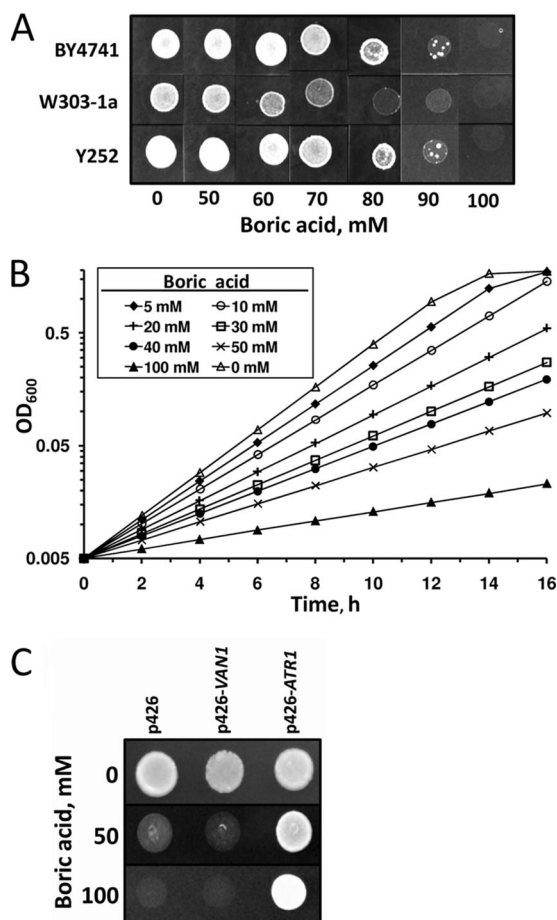


FIG. 1. Boric acid tolerance of yeast cells. (A) Three different wild-type strains were grown to logarithmic phase in rich (YPD) medium and diluted to an OD_{600} of 0.2, and 5 μ l of each cell suspension was spotted on plates containing the indicated amounts of boric acid. The plates were incubated at 30°C for 5 days and photographed. (B) An overnight culture of wild-type yeast (BY4741) was diluted to an OD_{600} of 0.05 and incubated with the indicated amounts of boric acid in YPD medium. Cell growth was monitored by OD_{600} measurements. (C) Wild-type cells (BY4741) were transformed with an empty vector (p426) or with p426-*VAN1* or p426-*ATR1* plasmid, and the transformants were spotted on YNB plates containing 0, 50, or 100 mM boric acid.

(Invitrogen). The W303-1a (*MATa ade2 ura3 leu2 trp1 his3 can1*) and Y252 (*MATa his3 leu2 met15 ura3-52 lys2-801 amber ade2,101ochre trp1 leu2*) strains were previously described by Wallis et al. (32) and Spector et al. (23), respectively. A single-step gene replacement procedure was applied to delete the *ATR1* gene in W303-1a cells. A disruption cassette containing the *URA3* marker gene was prepared using *Pfx* polymerase (Invitrogen) with the p426GAL vector as the template. Both primers contained homologous regions for recombination with the *ATR1* gene. The primers used were 5'-GTGTACGATTGTAAAAAGAGAGGCCAATTAGAGAATCTCAAACAGGTAATAACTTTTTTTTGGATCCGGTTTCTT TGA-3' and 5'-CGCATGACAGCGGTGTATTCTATCTATTACCTTAATAACCGCTTCCGCTAATTTGTGAGTTTAGTATACATGC-3'. The purified PCR fragment was transformed into the W303-1a strain using a standard lithium acetate method. Transformants were confirmed for deletion of *ATR1* by PCR using the primers *ATR1-F* (5'-TGGGCAATCAGTCATTAGTTGTGC) and *ATR1-B* (5'-TGCAATCGTATTCTGCAGCA). YPD medium (2% glucose, 2% peptone, 1% yeast extract, and 2% agar for solid medium) or yeast nitrogen base (YNB) medium (Bio101, Vista, CA) supplemented with the required amino acids or bases were used for cell growth.

Yeast genomic DNA library screening. The yeast genomic DNA library in YEpl3 was obtained from ATCC (no. 37323). Library plasmids were purified

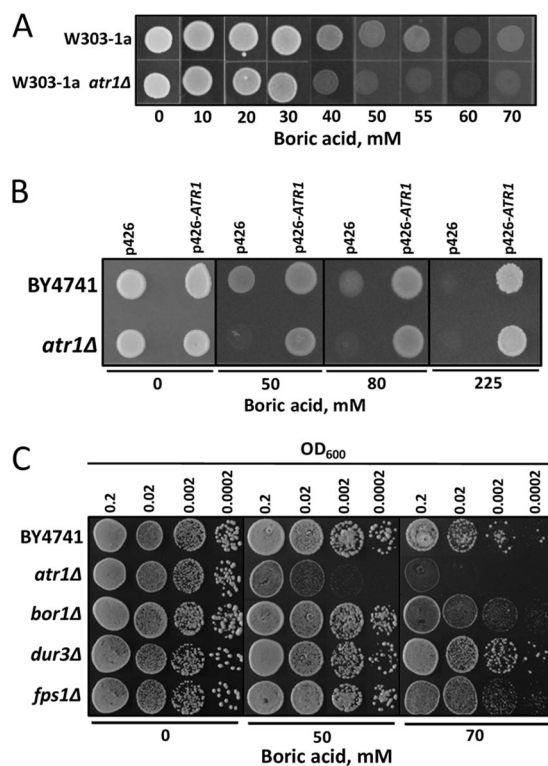


FIG. 2. The *ATR1* gene provides boron resistance. (A) Wild-type strain W303-1a and its isogenic *atr1Δ* mutant were grown to logarithmic phase in rich (YPD) medium, and the cultures were diluted to an OD_{600} of 0.2. Five microliters of each cell suspension was spotted on plates containing the indicated amounts of boric acid. The plates were incubated at 30°C for 5 days and photographed. (B) *ATR1*-overexpressing wild-type and *atr1Δ* cells were grown in the presence of the indicated concentrations of boron. Cells transformed with an empty vector were used as a control. (C) Comparisons of the boron tolerances of *atr1Δ*, *bor1Δ*, *dur3Δ*, and *fps1Δ* mutants. Overnight cultures were diluted to the indicated densities, and 5 μ l of each cell suspension was spotted. The cells were grown for 3 days, and the plates were photographed.

from *Escherichia coli* with a MaxiPrep kit (Invitrogen). Wild-type yeast (BY4741) was transformed with purified library DNA using the standard LiAc method, and cells were incubated on YNB medium containing 100 mM boric acid. Plates were incubated at 30°C for 5 days, and 30 colonies that grew in the presence of 100 mM boric acid were picked and regrown with higher concentrations of boron (125 to 200 mM) to confirm that the transformants were boron tolerant. Plasmids were isolated from selected clones and transferred to *E. coli* for amplification purposes. The isolated plasmids were then sequenced with vector-specific primers.

DNA microarray analysis. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.3 in 200 ml of YPD medium, with or without treatment for 1 hour with 20 mM boric acid; harvested by centrifugation; and kept at -80°C. Total RNA was isolated using the Ambion RiboPure yeast kit according to the manufacturer's instructions. The Agilent two-color low RNA input linear amplification kit was used to generate fluorescently labeled cRNA for two-color microarray hybridizations. Fluorescently labeled cRNA molecules were purified from the reaction mixture using the Qiagen RNeasy minikit. cRNA samples (825 ng each) were combined with Agilent Hi-RPM hybridization buffer. Microarray hybridizations were performed using Agilent SureHyb hybridization chambers. The hybridization chambers were loaded onto a rotisserie in an Agilent hybridization oven and were incubated at 65°C for 17 h with a rotational speed of 10 rpm. Following incubation, the microarray slides were washed for 1 min each in Gene Expression wash buffer 1 (6 \times SSPE [1 \times SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 , and 1 mM EDTA {pH 7.7}], 0.005% *N*-lauroylsarcosine at room temperature) and Gene Expression wash buffer 2 (0.06 \times SSPE, 0.005% *N*-

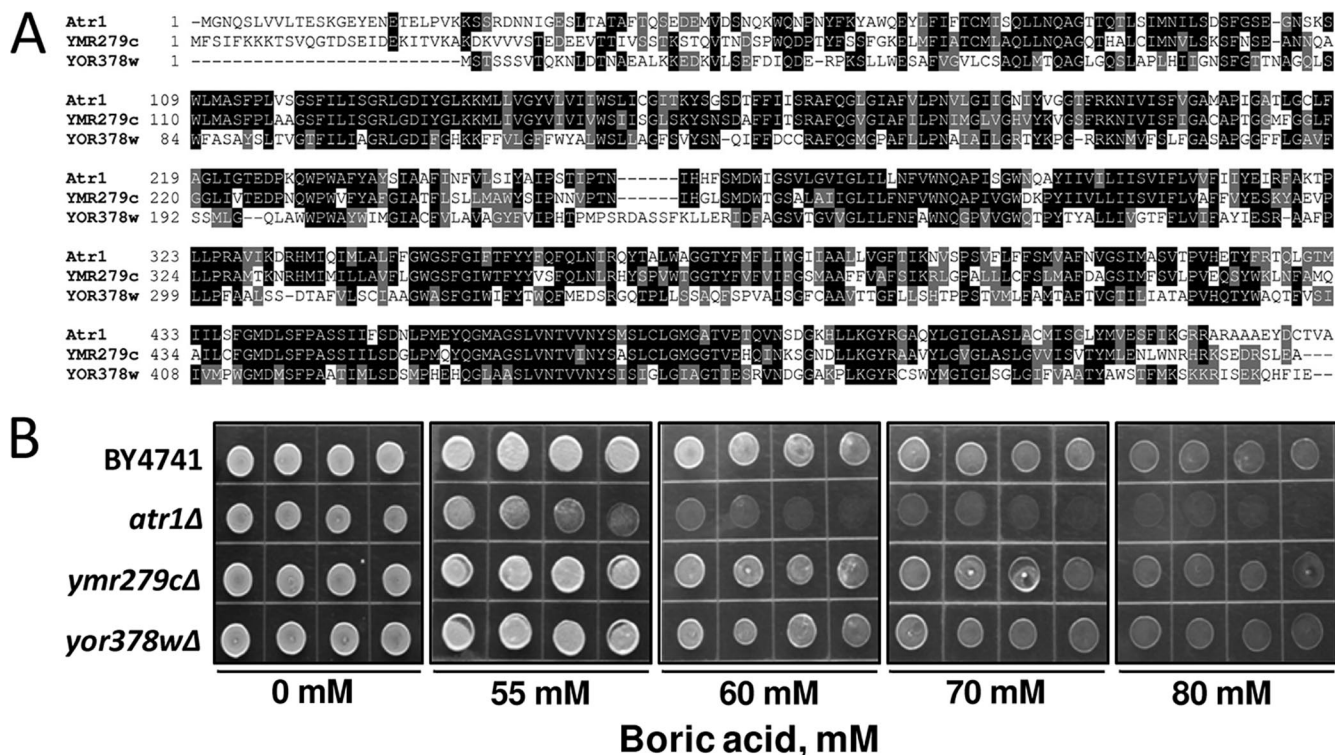


FIG. 3. Sequence alignment and roles of *ATR1* paralogs in boron tolerance. (A) Multiple-sequence alignment of yeast *ATR1* paralogs. Conserved residues are highlighted with BOXSHADE 3.21. (B) Comparisons of the boron tolerances of the *atr1Δ* mutant and paralogs, YMR279c and YOR378w mutants. Overnight cultures were diluted, and 5 μl of each cell suspension was spotted. The cells were grown for 3 days, and the plates were photographed.

lauroylsarcosine at 31°C) for 1 min each. The microarray slides were briefly dipped in a solution of acetonitrile, dried, and scanned in an Agilent Technologies G2505B microarray scanner at 5-μm resolution.

RNA isolation and real-time PCR analyses. Total RNA was isolated using the RNeasy minikit (Qiagen). Traces of genomic DNA were removed by DNase treatment (DNase RQ1; Promega). cDNA synthesis was performed using the First Strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. The cDNA was used as a template to amplify a 186-bp fragment for the *ATR1* gene and a 103-bp fragment for the internal control *ACT1* gene. Real-time PCR assays were performed with the IQ5 real-time PCR system (Bio-Rad). The primers used for amplification of *ATR1* were ATR1F (5'-ACGCGTATAGCATAGCCGCTTCA-3') and ATR1B (5'-TGTAAGCTGGTTCCAAACCGATA-3'), and those used for amplification of the *ACT1* gene were ACT1F (5'-ACGTCCAGCCTTCTACGTTTCCA-3') and ACT1B (5'-ACGTGAGTAACACCATCACCAGAA-3'). The conditions for PCR amplification were as follows: 30 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s.

Overexpression of the *ATR1* gene. The wild-type allele of the *ATR1* gene was amplified by *Pfu* polymerase (Fermentas) from yeast genomic DNA using the primers ATR1F (5'-TGGATCCGAGGCAATTAGAGAATCTCAAACAGG-3') and ATR1B (5'-ACTCGAGCTTCCGCTAAGCCACAGTGCAATCG-3') containing BamHI and XhoI restriction sites, respectively. The amplified fragment was first cloned into the SmaI site of pBluescript II KS (Stratagene) and then moved into the BamHI/XhoI sites of the yeast high-copy-number expression vector p426GPD to generate a p426-*ATR1* expression construct. Yeast cells were transformed with the empty vector p426 or with p426-*ATR1* and selected for uracil prototrophy.

Determination of intracellular boron concentrations. Exponentially growing cells were treated with 50 mM boric acid for 1 hour and harvested by centrifugation. The cells were washed with distilled water three times and either kept at -80°C or 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) using a vortex. The supernatants were transferred to new tubes, and the protein concentrations were determined by the Bradford protein detection assay (2). The boron concentra-

tions of diluted cell extracts were determined using the boron cell test kit (Merck, Germany) as described in the manufacturer's instructions and normalized by the amount of total protein in the cell extracts. The measurement sensitivity of the kit was 8×10^{-7} M (0.05 mg boron/liter) as stated by the manufacturer.

Protein localization. A previously described procedure (13) was followed for tagging the chromosomal *ATR1* gene with GFP at the C terminus. Primers 116S3 (5'-AGGCCGAGGCAAGAGCTGCTGCAGAATACGATTGCACTGTGCTCTGATCTGCAGGTCGAC-3') and 116S2 (5'-CATGACAGCGGTGATTCTATCTATTACCTTAATAACCGCTTCCGATCGATGAATCCGAGCTCG-3') were used to amplify a cassette including GFP and the *Kluyveromyces lactis TRP1* marker from plasmid pMY26 (13). The resulting PCR fragment was used to transform W3031-a (*MATa ade2 ura3 leu2 trp1 his3*) (32) wild-type cells, and transformants were selected on YNB-uracil dropout medium. Exponentially growing cells were analyzed with a confocal fluorescence microscope.

Western blot analysis. Wild-type cells (W3031-a) and the isogenic *ATR1-GFP* strains were grown in YPD medium to logarithmic phase, and equal numbers of cells were harvested by centrifugation for each strain. Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotting. The GFP signal was detected by primary and secondary antibodies from Sigma and SuperSignal West Pico chemiluminescent substrate from Pierce.

Comparative genomic analyses of *ATR1*, *BOR1*, *DUR3*, and *FPS1* in prokaryotes and eukaryotes. Sequenced genomes of 45 archaea and 547 bacteria and 53 annotated genomes of eukaryotes (37 completely sequenced and 16 unfinished) from NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) were analyzed for the occurrence of boron transporter genes. *S. cerevisiae* Atr1 protein (gi 798884) was used as the query in TBLASTN and BLASTP searches with default parameters. Orthologous proteins were defined by a bidirectional BLAST test. Hits were considered orthologous if *S. cerevisiae* Atr1 was among the three best hits in bidirectional BLAST tests with an E value of $1e-5$.

Multiple-sequence alignments. Multiple-sequence alignments were performed using CLUSTALW and T-Coffee with the default parameters. Phylogenetic trees were prepared using the Interactive Tree Of Life service (<http://itol.embl.de/>), which is based on the highly resolved tree of life project (3).

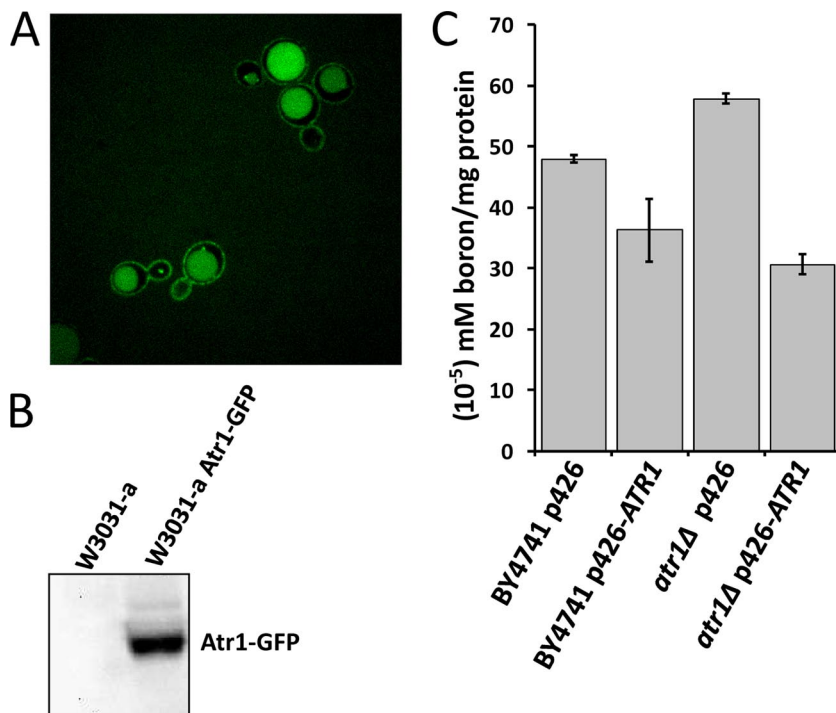


FIG. 4. Atr1 localization and boron efflux function. (A) Atr1 localizes to the cell membrane and vacuole. The *ATR1* gene was tagged with GFP at the C terminus, and cells expressing the Atr1-GFP fusion protein were photographed with a confocal fluorescence microscope. (B) Western blot analysis of the Atr1-GFP fusion protein with anti-GFP antibody showing a single band. (C) Intracellular boron levels in wild-type and *atr1Δ* cells that were exposed to 50 mM boric acid for 1 h. The cells were transformed with either empty vector or an *ATR1* overexpression plasmid (p426-*ATR1*). Boron levels were normalized to protein levels. The error bars represent three separate measurements.

RESULTS

Identification of *ATR1* as a boron resistance gene. We first tested several wild-type strains for boron tolerance and found that cells did not grow well at concentrations of boric acid above 80 mM (Fig. 1A and B). We used this observation to screen a high-copy-number yeast genomic DNA library for genes that confer boron resistance on BY4741 wild-type cells. Transformed cells were exposed to 100 mM boric acid for 5 days. Thirty colonies grew in the presence of 100 mM boric acid. Each colony was regrown on plates with increasing amounts of boric acid. All identified colonies grew in the presence of 200 mM boric acid. We recovered plasmids from five randomly chosen colonies and sequenced the inserts using a pair of vector-based primers. All of the inserts had sequences derived from *S. cerevisiae* chromosome XIII, spanning a region that contained intact *VANI* (YML115c) and *ATR1* (YML116w) genes. PCR analyses of the other library constructs showed that they also contained the same region of chromosome XIII. Van1 plays a role in mannan synthesis (24) and vanadium tolerance (12). Atr1 is a drug-H⁺ antiporter of the major facilitator superfamily with unknown function (7, 11).

To determine whether *VANI* or *ATR1* conferred boron resistance on cells, the two genes were separately cloned into the yeast high-copy-number expression vector p426GPD and expressed in BY4741 wild-type cells. Overexpression of the *ATR1* gene provided strong boron resistance (Fig. 1C), whereas overexpression of *VANI* did not. We concluded that the *ATR1* gene was responsible for boron tolerance.

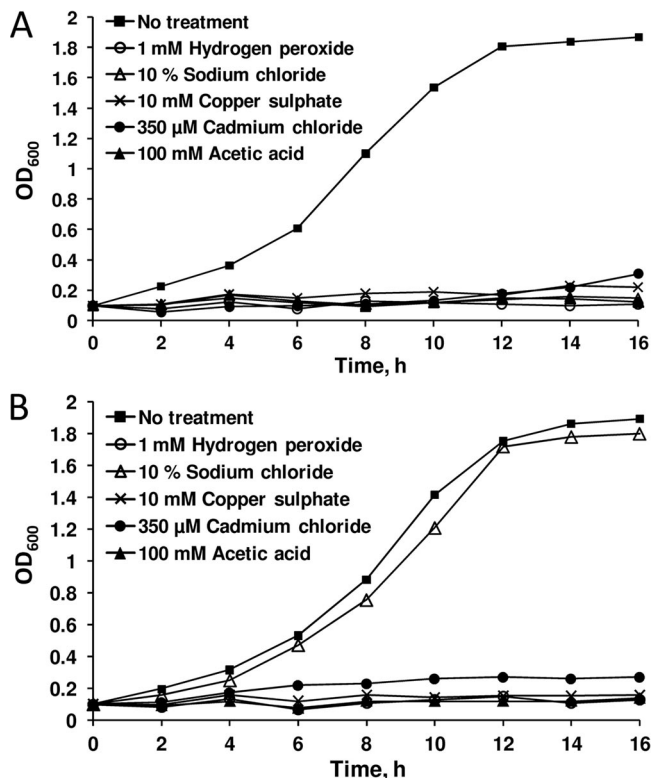


FIG. 5. Role of *ATR1* in yeast stress tolerance. Wild-type cells were transformed with p426 (A) and p426-*ATR1* (B), and the transformants were grown in the presence of the indicated toxic substances. Growth rates were determined by OD₆₀₀ measurements.

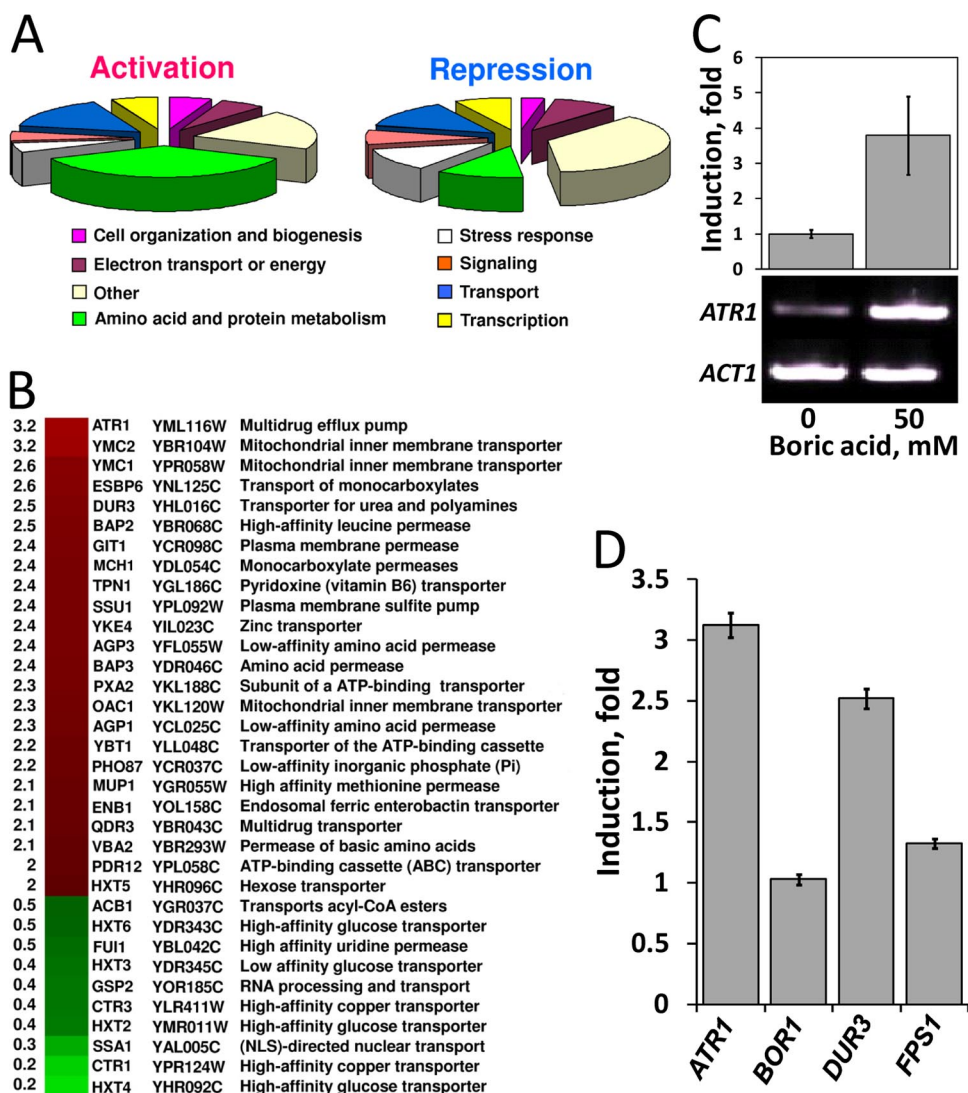


FIG. 6. Genomic expression analyses of boron-treated cells. Logarithmically growing cells were split into two groups, one of which was treated with 20 mM boric acid for 1 hour while the other was not. The gene expression patterns of the two groups were then compared. (A) Functional categories of boron-responsive genes with known functions. Genes activated or repressed at least twofold are shown. (B) Transporter genes that were upregulated or downregulated at least threefold in response to boron treatment. (C) Real-time PCR transcriptional analysis of *ATR1* in response to boron treatment. Wild-type cells were treated or not with 50 mM boric acid, and *ATR1* mRNA levels were detected (top). PCR products were run on a 1% Tris-acetate-EDTA-agarose gel (bottom). Actin (*ACT1*) was used as the internal control. (D) Transcriptional regulation of *ATR1*, *BOR1*, *DUR3*, and *FPS1* genes by boron treatment. The data are derived from microarray experiments. The error bars represent four replicates.

Next, we tested whether deletion of *ATR1* leads to boron sensitivity. As shown in Fig. 2A and B, *atr1Δ* mutants in both the W3031-a and BY4741 backgrounds did not grow in the presence of boric acid in excess of 50 mM, whereas wild-type cells tolerated larger amounts of boric acid. Overexpression of *ATR1* in the *atr1Δ* mutant enabled cell growth in the presence of boron. In fact, *ATR1* expression provided very strong boron resistance to both *atr1Δ* and wild-type cells, as they could grow in the presence of 225 mM boric acid (Fig. 2B).

To determine the overall contribution of *ATR1* to boron toxicity resistance, we compared the boron tolerance of *atr1Δ* mutants to those of *bor1Δ*, *dur3Δ*, and *fps1Δ* mutants. These

mutants are defective for the yeast genes that were previously reported to be involved in boron resistance. The boron tolerances of the four mutants were compared by growing cells on boron-containing medium (Fig. 2C). The *atr1Δ* mutant was the most sensitive to boron treatment. Its growth was inhibited by 50 mM boric acid, whereas the growth of the other mutants was unaffected. Thus, deletion of the *ATR1* gene created stronger boron sensitivity than deletion of any other known boron resistance gene.

***ATR1* paralogs in the *S. cerevisiae* genome.** Two *ATR1* paralogs were identified in the *S. cerevisiae* genome by sequence analyses (Fig. 3A). *Atr1* and its paralogs share a pfam07690 domain and show more than 70% identity in their sequences.

Strains with YMR279c and YOR378w deletions were tested for boron tolerance, but no difference from wild-type cells was found, suggesting that, among the three paralogs, only *ATR1* provides boron resistance (Fig. 3B).

Atr1 localization. Atr1 is a predicted membrane protein containing 14 transmembrane segments (7, 8). We tagged the native *ATR1* gene with a GFP tag and examined the localization of the fusion protein. Confocal microscopy analysis showed that the Atr1-GFP fusion protein localized to the plasma membrane and vacuole (Fig. 4A). To confirm that the vacuolar localization of the fusion protein was not the result of degradation of the intact protein, we performed Western blot analysis using an antibody against GFP. As seen in Fig. 4B, there was only one band for the Atr1-GFP fusion protein, suggesting that the protein was intact.

Atr1 is a boron efflux transporter. To examine the mechanism of boron resistance by Atr1, we determined the intracellular boron levels in wild-type and *atr1Δ* cells. Exponentially growing cells were treated with 50 mM boric acid for 1 hour, and then their intracellular boron levels were determined. As shown in Fig. 4C, *atr1Δ* cells had 21% more boron than wild-type cells. Overexpression of *ATR1* lowered the intracellular boron concentration by 25% in wild-type cells and by 47% in *atr1Δ* cells. Thus, the absence of the *ATR1* gene caused accumulation of boron inside the cells, whereas its overexpression led to the exclusion of boron from the cells.

To check the possibility that Atr1 might be a general stress tolerance protein with multiple substrates, we tested wild-type cells that overexpressed *ATR1* for different stress conditions, including oxidative stress, salt stress, heavy metal stress, and acetic acid stress (Fig. 5). Among these tested conditions, *ATR1* overexpression provided resistance only to salt stress.

Transcriptional response to boron treatment. We analyzed the transcriptional response of wild-type yeast cells to 20 mM boric acid using DNA microarrays. Interestingly, groups of genes involved in amino acid biosynthesis and membrane transport were differentially expressed (Fig. 6A). Among transporter genes whose expression was induced by boric acid, *ATR1* had the greatest increase (over threefold induction) (Fig. 6B). Expression of the *ATR1* paralogs YMR279C and YOR378W was induced by 1.4- and 1.6-fold, respectively. Upregulation of the *ATR1* gene in response to boron treatment was also observed by real-time PCR. *ATR1* transcripts showed fourfold-higher expression in cells exposed to 50 mM boric acid for 1 hour than in control cells (Fig. 6C). Expression levels of the *BORI* and *FPS1* genes did not change in response to boron treatment, but *DUR3* was upregulated 2.5-fold (Fig. 6D). Thus, our expression analyses showed that the *ATR1* gene is more strongly upregulated in the presence of boron than the three previously known boron transporters.

In addition to *ATR1*, transcription of the *QDR3* (YBR043c) gene, whose product is a member of the DHA1 family of drug-H⁺ antiporters with 12 predicted membrane-spanning segments, was upregulated twofold in response to boron in the microarray analysis (Fig. 6B). To test whether the *QDR3* gene plays a role in boron tolerance similar to that of *ATR1*, we cloned and overexpressed it in wild-type (BY4741) and *qdr3Δ* mutant cells. As seen in Fig. 7, neither overexpression nor deletion of the *QDR3* gene changed the boron tolerance level of the cells.

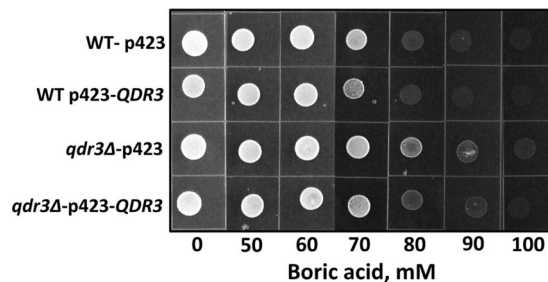


FIG. 7. Deletion or overexpression of *QDR3* has no effect on boron tolerance. Wild-type (WT) strain BY4741 and its isogenic *qdr3Δ* mutant were transformed with an empty vector and p423-*QDR3* plasmid. The transformants were grown to logarithmic phase in YNB-His medium, and the cultures were diluted to an OD₆₀₀ of 0.2. Five microliters of each cell suspension was spotted on plates containing the indicated amounts of boric acid. The plates were incubated at 30°C for 5 days and photographed.

To better understand the regulation of *ATR1*, *BORI*, *DUR3*, *FPS1*, and the two *ATR1* paralogs, YMR279c and YOR378w, we analyzed their expression changes in response to various stresses (Fig. 8) and participation in the yeast environmental stress response (6). There was significant upregulation of *ATR1* expression in response to hydrogen peroxide, diamide, and menadione, whereas the expression of the other boron transporters was not changed.

Widespread occurrence of *ATR1* homologs in fungi, bacteria, and archaea. *ATR1* homologs were detected in fungi, bacteria, and archaea (Fig. 9; see Fig. S1 in the supplemental material), but not in animals, vascular plants, and algae. Vascular plants and *Dictyostelium discoideum* contained distantly related *ATR1* homologs with unknown functions. Actinobacteria had the highest content of *ATR1* orthologs among bacteria and *Sulfolobales* among archaea. *ATR1* distribution in eukaryotes was limited to fungi. We also analyzed the occurrence of other boron transporters in the three domains of life. *FPS1* and *DUR3* homologs showed scattered occurrence, whereas *BORI* was mainly found in eukaryotes.

DISCUSSION

We identified *ATR1* as a major boron resistance gene in yeast by screening a genomic DNA library for genes that support the growth of yeast cells in the presence of high concentrations of boric acid. Two *ATR1* paralogs, YMR279c and YOR378w, could not be linked to boron detoxification, as the corresponding mutants were not boron sensitive. Overexpression of *ATR1* provided boron resistance to wild-type cells, and its deletion reduced boron tolerance levels. In boron-containing media, the intracellular boron concentration of *atr1Δ* mutants was higher than that of wild-type cells, whereas overexpression of the *ATR1* gene reduced intracellular boron levels. We conclude that Atr1 functions as a boron efflux transporter and helps cells to keep intracellular boron below toxic levels.

Several genes have been shown to have roles in boron resistance in yeast. Among these, the functions of *DUR3* and *FPS1* in boron export are not clear (20). *DUR3* and *FPS1* deletion mutants did not show elevated levels of intracellular boron; however, deletion of *BORI* increased boron levels in yeast cells

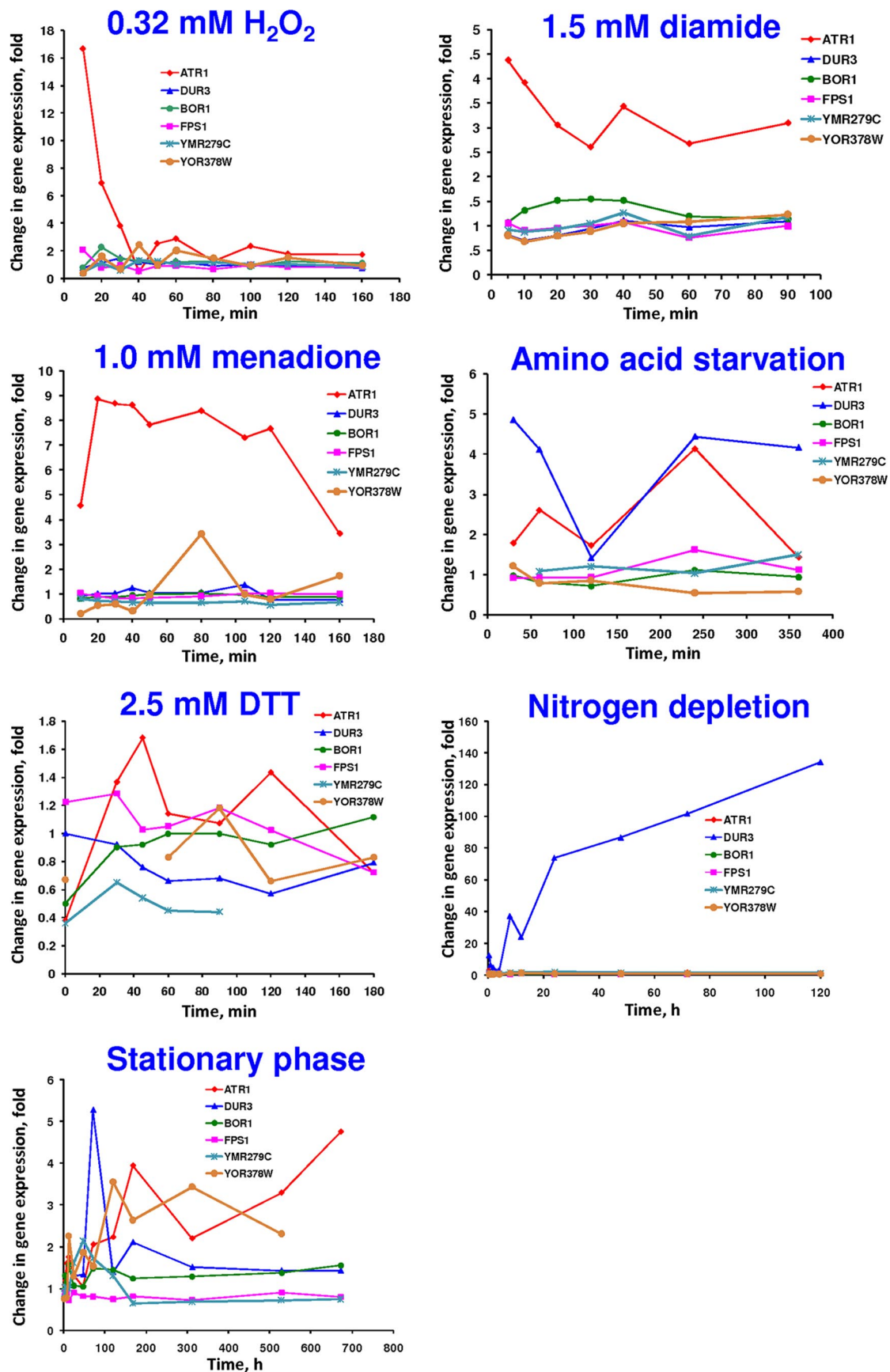


FIG. 8. Time course of changes in expression of the boron transporter gene and *ATR1* paralogs in response to the indicated chemical stressors, 0.32 mM H₂O₂, 1.5 mM diamide, 1 mM menadione, and 2.5 mM dithiothreitol (DTT), and to the indicated stress conditions, amino acid starvation, nitrogen depletion, and stationary phase.

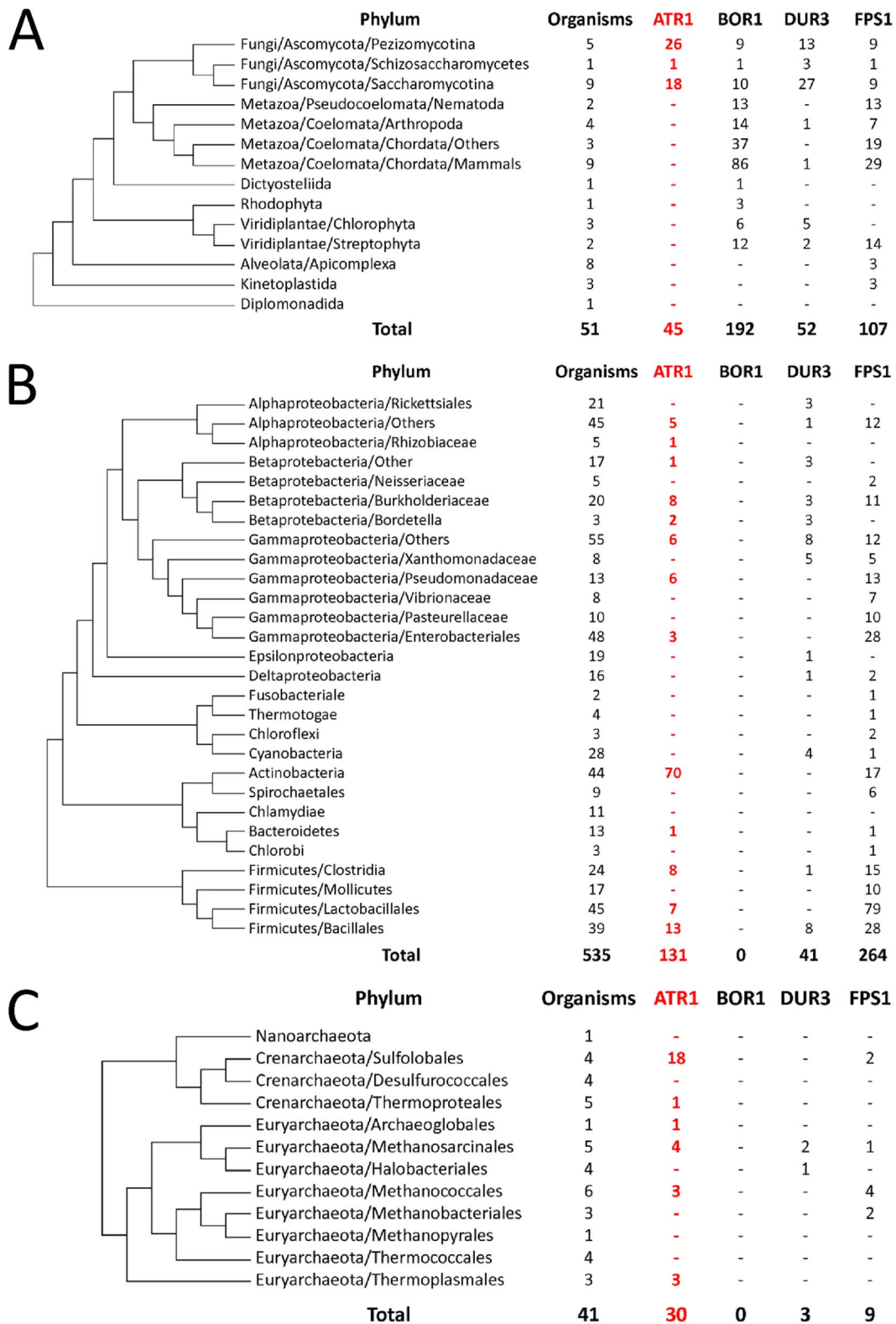


FIG. 9. Occurrence of *ATR1* in the three domains of life. Distribution of *ATR1* in *Eukaryota* (A), *Bacteria* (B), and *Archaea* (C). The phylogenetic trees were derived from a highly resolved tree of life (3).

(20). Overexpression of the *BOR1* and *FPS1* genes resulted in lower cytoplasmic boron levels, while overexpression of *DUR3* resulted in slightly increased amounts of boron (20). Among all boron transporters, Atr1 showed the widest phylogenetic distribution (Fig. 9; see Fig. S1 in the supplemental material). The diverse occurrence of these boron transporters (characterized by unrelated protein sequences) suggests the possibility of independent evolution of their functions in boron detoxification.

Our microarray data showed that expression of the *BOR1* and *FPS1* genes did not change in response to boron treatment, while *DUR3* was upregulated 2.5-fold (Fig. 6D). Jennings et al. also showed that *BOR1* transcription is not induced by exposure of cells to boron (10). We compared the growth rates of *atr1Δ*, *bor1Δ*, *fps1Δ*, and *dur3Δ* mutants in the presence of boron and found that only deletion of the *ATR1* gene led to boron hypersensitivity (Fig. 2C), suggesting that *ATR1* has the most critical function in eliminating boron. The *atr1Δ* mutant was hypersensitive to boron treatment, and cells overexpressing *ATR1* survived in the presence of 225 mM boric acid, which is the largest amount of boron shown to be tolerated by a eukaryotic system. *ATR1* expression was also significantly upregulated by boron exposure (Fig. 6). Thus, *ATR1* is a physiologically relevant boron tolerance gene in yeast. Larger amounts of intracellular boron in *atr1Δ* mutants and smaller amounts in *ATR1*-overexpressing cells suggest that Atr1 controls boron traffic in one direction, toward the extracellular space.

Transcriptional control of *ATR1* has not been studied in detail, but it is known that Gcn4 and Yap1 transcription factors play roles in *ATR1* induction (4). Apart from the other boron tolerance genes, *ATR1* expression was upregulated in response to hydrogen peroxide, diamide, and menadione treatments (Fig. 8), which could be explained by a Yap1-dependent mechanism. Yap1 is a transcription factor that regulates expression of oxidative-stress response genes (15). The expression patterns of the *ATR1* and *DUR3* genes in response to amino acid starvation were similar (Fig. 8).

ATR1 could also be viewed as a stress tolerance gene in lower eukaryotes and prokaryotes; however, except for boron, its substrate spectrum is not clear. The role of Atr1 in 3-amino-1,2,4-triazole and 4-nitroquinoline-*N*-oxide resistance was shown previously. To investigate the possibility that the *ATR1* product may be a general stress tolerance protein with multiple substrates besides boron, we subjected wild-type cells that overexpressed *ATR1* to different stress conditions (Fig. 5). *ATR1* overexpression provided resistance to salt stress but not to oxidative, heavy metal, or acetic acid stress. Thus, although *ATR1* may not be a general stress tolerance gene, it may have a role in the detoxification of more than one substrate.

In summary, four properties of *ATR1* provided evidence for its role in boron resistance: (i) deletion of *ATR1* led to boron sensitivity, (ii) overexpression of *ATR1* increased boron tolerance, (iii) *ATR1* expression was regulated by boron, and (iv) the concentration of intracellular boron was controlled by *ATR1*. Compared to other proteins previously shown to play roles in boron metabolism in yeast, Atr1 emerges as the most effective.

ACKNOWLEDGMENTS

We thank Karim Labip (Paterson Institute for Cancer Research, United Kingdom) for providing the pMY12 vector, Serdar Özçelik (Izmir Institute of Technology, Izmir, Turkey) for his help with confocal fluorescence microscopy, and the biotechnology core facility of the Izmir Institute of Technology for help with instruments. We also thank Anne Fray for critical review of the manuscript and Irem Uluisik, Banu Demir, and Elise Hacıoglu for their help with some of the experiments.

This work was supported by Turkish Scientific and Technical Research Council (TUBITAK) Grant no. 104T213 and a Turkish Academy of Science GEBIP grant to A.K. and in part by NIH grants to V.N.G. Alaattin Kaya was supported by the Graduate Student Support Program of TUBITAK-BIDEP.

REFERENCES

- Bassil, E., H. Hu, and P. H. Brown. 2004. Use of phenylboronic acids to investigate boron function in plants. Possible role of boron in transvacuolar cytoplasmic strands and cell-to-wall adhesion. *Plant Physiol.* **136**:3383–3395.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Ciccarelli, F. D., T. Doerks, C. von Mering, C. J. Creevey, B. Snel, and P. Bork. 2006. Toward automatic reconstruction of a highly resolved tree of life. *Science* **311**:1283–1287.
- Coleman, S. T., E. Tseng, and W. S. Moye-Rowley. 1997. *Saccharomyces cerevisiae* basic region-leucine zipper protein regulatory networks converge at the *ATR1* structural gene. *J. Biol. Chem.* **272**:23224–23230.
- ElBerry, H. M., M. L. Majumdar, T. S. Cunningham, R. A. Sumrada, and T. G. Cooper. 1993. Regulation of the urea active transporter gene (*DUR3*) in *Saccharomyces cerevisiae*. *J. Bacteriol.* **175**:4688–4698.
- Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown. 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11**:4241–4257.
- Gbelska, Y., J. J. Krijger, and K. D. Breunig. 2006. Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. *FEMS Yeast Res.* **6**:345–355.
- Goffeau, A., J. Park, I. T. Paulsen, J. L. Jonniaux, T. Dinh, P. Mordant, and M. H. Saier, Jr. 1997. Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames with the major facilitator superfamily. *Yeast* **13**:43–54.
- Iwai, H., A. Hokura, M. Oishi, H. Chida, T. Ishii, S. Sakai, and S. Satoh. 2006. The gene responsible for borate cross-linking of pectin rhamnogalacturonan-II is required for plant reproductive tissue development and fertilization. *Proc. Natl. Acad. Sci. USA* **103**:16592–16597.
- Jennings, M. L., T. R. Howren, J. Cui, M. Winters, and R. Hannigan. 2007. Transport and regulatory characteristics of the yeast bicarbonate transporter homolog Bor1p. *Am. J. Physiol. Cell Physiol.* **293**:C468–C476.
- Kanazawa, S., M. Driscoll, and K. Struhl. 1988. *ATR1*, a *Saccharomyces cerevisiae* gene encoding a transmembrane protein required for aminotriazole resistance. *Mol. Cell. Biol.* **8**:664–673.
- Kanik-Ennulat, C., and N. Neff. 1990. Vanadate-resistant mutants of *Saccharomyces cerevisiae* show alterations in protein phosphorylation and growth control. *Mol. Cell. Biol.* **10**:898–909.
- Knop, M., K. Siegers, G. Pereira, W. Zachariae, B. Winsor, K. Nasmyth, and E. Schiebel. 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15**:963–972.
- Kobayashi, M., T. Matoh, and J. Azuma. 1996. Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiol.* **110**:1017–1020.
- Lee, J., C. Godon, G. Lagniel, D. Spector, J. Garin, J. Labarre, and M. B. Toledano. 1999. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* **274**:16040–16046.
- Luyten, K., J. Albertyn, W. F. Skibbe, B. A. Prior, J. Ramos, J. M. Thevelein, and S. Hohmann. 1995. Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.* **14**:1360–1371.
- Mack, M., P. Gompel-Klein, E. Haase, J. Hietkamp, A. Ruhland, and M. Brendel. 1988. Genetic characterization of hyperresistance to formaldehyde and 4-nitroquinoline-*N*-oxide in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **211**:260–265.
- Miwa, K., J. Takano, H. Omori, M. Seki, K. Shinozaki, and T. Fujiwara. 2007. Plants tolerant of high boron levels. *Science* **318**:1417.
- Nozawa, A., K. Miwa, M. Kobayashi, and T. Fujiwara. 2006. Isolation of *Arabidopsis thaliana* cDNAs that confer yeast boric acid tolerance. *Biosci. Biotechnol. Biochem.* **70**:1724–1730.
- Nozawa, A., J. Takano, M. Kobayashi, N. von Wiren, and T. Fujiwara. 2006.

- Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **262**:216–222.
21. O'Neill, M. A., T. Ishii, P. Albersheim, and A. G. Darvill. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu. Rev. Plant Biol. **55**:109–139.
 22. Park, M., Q. Li, N. Shcheynikov, W. Zeng, and S. Muallem. 2004. NaBC1 is a ubiquitous electrogenic Na⁺-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation. Mol. Cell **16**:331–341.
 23. Spector, D., J. Labarre, and M. B. Toledano. 2001. A genetic investigation of the essential role of glutathione: mutations in the proline biosynthesis pathway are the only suppressors of glutathione auxotrophy in yeast. J. Biol. Chem. **276**:7011–7016.
 24. Stolz, J., and S. Munro. 2002. The components of the *Saccharomyces cerevisiae* mannosyltransferase complex M-Pol I have distinct functions in mannan synthesis. J. Biol. Chem. **277**:44801–44808.
 25. Sutton, T., U. Baumann, J. Hayes, N. C. Collins, B. J. Shi, T. Schnurbusch, A. Hay, G. Mayo, M. Pallotta, M. Tester, and P. Langridge. 2007. Boron toxicity tolerance in barley arising from efflux transporter amplification. Science **318**:1446–1449.
 26. Takano, J., M. Kobayashi, Y. Noda, and T. Fujiwara. 2007. *Saccharomyces cerevisiae* Bor1p is a boron exporter and a key determinant of boron tolerance. FEMS Microbiol. Lett. **267**:230–235.
 27. Takano, J., K. Miwa, L. Yuan, N. von Wiren, and T. Fujiwara. 2005. Endocytosis and degradation of BOR1, a boron transporter of *Arabidopsis thaliana*, regulated by boron availability. Proc. Natl. Acad. Sci. USA **102**:12276–12281.
 28. Takano, J., K. Noguchi, M. Yasumori, M. Kobayashi, Z. Gajdos, K. Miwa, H. Hayashi, T. Yoneyama, and T. Fujiwara. 2002. *Arabidopsis* boron transporter for xylem loading. Nature **420**:337–340.
 29. Takano, J., M. Wada, U. Ludewig, G. Schaaf, N. von Wiren, and T. Fujiwara. 2006. The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell **18**:1498–1509.
 30. Tamas, M. J., K. Luyten, F. C. Sutherland, A. Hernandez, J. Albertyn, H. Valadi, H. Li, B. A. Prior, S. G. Kilian, J. Ramos, L. Gustafsson, J. M. Thevelein, and S. Hohmann. 1999. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol. Microbiol. **31**:1087–1104.
 31. Uemura, T., K. Kashiwagi, and K. Igarashi. 2007. Polyamine uptake by DUR3 and SAM3 in *Saccharomyces cerevisiae*. J. Biol. Chem. **282**:7733–7741.
 32. Wallis, J. W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein. 1989. A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. Cell **58**:409–419.
 33. Wysocki, R., C. C. Chery, D. Wawrzycka, M. Van Hulle, R. Cornelis, J. M. Thevelein, and M. J. Tamas. 2001. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. Mol. Microbiol. **40**:1391–1401.