Identification of a Novel System for Boron Transport: Atr1 Is a Main Boron Exporter in Yeast

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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 *ATR1* (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 *ATR1* gene expression was upregulated by boron and several stress conditions. We found that *atr1*Δ mutants were highly sensitive to boron treatment, whereas cells overexpressing *ATR1* were boron resistant. In addition, *atr1*Δ cells accumulated boron, whereas *ATR1*-overexpressing cells had low intracellular levels of the element. Furthermore, *atr1*Δ cells showed stronger boron-dependent phenotypes than mutants deficient in genes previously reported to be implicated in boron metabolism. *ATR1* 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 rhammogalacturanon 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 boron acid exporter (26). The *bor1*Δ 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 antimite 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. *ATR1* 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 *gen4Δ* 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 *ATR1* conferred extreme resistance to boron and reduced intracellular levels of the element, whereas cells lacking the *ATR1* 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 *ATR1* 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.

**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.
acetate method. Transformants were confirmed for deletion of the ATR1 gene in W303-1a cells. A disruption cassette containing the URA3 marker gene was prepared using PCR amplification with the p426GAL vector as the template. Both primers contained homologous regions for recombination with the ATR1 gene. The primers used were 5'-GTGTACGATTGTAAAAAGAGAGCAGTA-3' and 5'-CGCATGACAGCGGTGTATTTCTATTTACCTTAATAACGCCTTTCCGCTAATTTGTGAGTTTAGTATACATGC-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'-GGGCAATCAGTCATTGTTGTCG-3') and ATR1-R (5'-TGGCAATCGTAAATCATCGCA-3').

**Yeast genomic DNA library screening.** The yeast genomic DNA library in YEpl3 was obtained from ATCC (no. 37323). Library plasmids were purified 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 (OD600) 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× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA (pH 7.7)], 0.005% N-lauroylsarcosine at room temperature) and Gene Expression wash buffer 2 (0.06× SSPE, 0.005% N-
Atr1 is a boron efflux pump.

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.

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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 VAN1 (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^+/H^+ antiporter of the major facilitator superfamily with unknown function (7, 11).

To determine whether VAN1 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 VAN1 did not. We concluded that the ATR1 gene was responsible for boron tolerance.

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.

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 OD_{600} measurements.
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. Up-regulation 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 BOR1 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.

**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 BOR1 increased boron levels in yeast cells.

To better understand the regulation of ATR1, BOR1, 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 BOR1 was mainly found in eukaryotes.
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 H$_2$O$_2$, 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).
Overexpression of the \textit{BOR1} and \textit{FPS1} genes resulted in lower cytoplasmic boron levels, while overexpression of \textit{DUR3} resulted in slightly increased amounts of boron (20). Among all boron transporters, Atr1 showed the widest phyletic 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 \textit{BOR1} and \textit{FPS1} genes did not change in response to boron treatment, while \textit{DUR3} was upregulated 2.5-fold (Fig. 6D). Jennings et al. also showed that \textit{BOR1} transcription is not induced by exposure of cells to boron (10). We compared the growth rates of \textit{atr1Δ}, \textit{bor1Δ}, \textit{fps1Δ}, and \textit{dur3Δ} mutants in the presence of boron and found that only deletion of the \textit{ATR1} gene led to boron hypersensitivity (Fig. 2C), suggesting that \textit{ATR1} has the most critical function in eliminating boron. The \textit{atr1Δ} mutant was hypersensitive to boron treatment, and cells overexpressing \textit{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. \textit{ATR1} expression was also significantly up-regulated by boron exposure (Fig. 6). Thus, \textit{ATR1} is a physiologically relevant boron tolerance gene in yeast. Larger amounts of intracellular boron in \textit{at1Δ} mutants and smaller amounts in \textit{ATR1}-overexpressing cells suggest that Atr1 controls boron traffic in one direction, toward the extracellular space.

Transcriptional control of \textit{ATR1} has not been studied in detail, but it is known that Gcn4 and Yap1 transcription factors play roles in \textit{ATR1} induction (4). Apart from the other boron tolerance genes, \textit{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 \textit{ATR1} and \textit{DUR3} genes in response to amino acid starvation were similar (Fig. 8).

\textit{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 \textit{ATR1} product may be a general stress tolerance protein with multiple substrates besides boron, we subjected wild-type cells that overexpressed \textit{ATR1} to different stress conditions (Fig. 5). \textit{ATR1} overexpression provided resistance to salt stress but not to oxidative, heavy metal, or acetic acid stress. Thus, although \textit{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 \textit{ATR1} provided evidence for its role in boron resistance: (i) deletion of \textit{ATR1} led to boron sensitivity, (ii) overexpression of \textit{ATR1} increased boron tolerance, (iii) \textit{ATR1} expression was regulated by boron, and (iv) the concentration of intracellular boron was controlled by \textit{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, 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 Frary for critical review of the manuscript and Irem Ulusik, Banu Demir, and Elise Hacioglu 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. Alaaatyn Kaya was supported by the Graduate Student Support Program of TUBITAK-BIDEP.

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