Proteomic changes during boron tolerance in barley
(*Hordeum vulgare*) and the role of vacuolar
proton-translocating ATPase subunit E

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**Abstract:** Boron is an essential micronutrient for plants and animals; however, it can be toxic when present at high concentrations. The purpose of this study was to understand the mechanisms of boron tolerance in the Turkish barley (*Hordeum vulgare*) Anadolu cultivar. For this purpose, 2-dimensional electrophoresis (2-DE) was used to screen differentially expressed proteins for both control and boron-stressed Anadolu barley genotypes. Seven proteins were revealed by 2-DE: 1) ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo large chain), 2) TLP5, a thaumatin-like protein, 3) PR3, a basic pathogenesis-related protein, 4) a RNase S-like protein, 5) a PSI type III chlorophyll a/b-binding protein, 6) a light-harvesting complex I LHC I, and 7) the vacuolar proton-translocating ATPase subunit E protein. These were found to be upregulated in response to boron treatment. Even though the protein encoded by the V-ATPase subunit E gene was overexpressed, its transcript level was downregulated by boron treatment. Heterologous expression of the barley V-ATPase subunit E gene in yeast provided boron resistance to yeast cells. These results indicated that the V-ATPase subunit E gene was functional and conferred tolerance to toxic boron levels in yeast and might play a role in the overall boron tolerance of barley.

**Key words:** Boron stress, *Hordeum vulgare*, *Saccharomyces cerevisiae*, proteomics, V-ATPase

Boron toleransı sırasında arpada (*Hordeum vulgare*) görülen proteomik
değişimler ve vaküol proton transloksasyon ATPaz alt birim E’nin rolü


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Introduction

Boron is an essential micronutrient for both plants and animals. Despite its essentiality for higher plants, high boron concentrations in soil limit crop production all over the world (Sutton et al., 2007). In that context, understanding boron toxicity mechanisms and identifying boron tolerance genes in crop species is very important. Boron functions as a cross-linker for rhamnogalacturonan-II in the cell membrane (Kobayashi et al., 1996; Ishii et al., 1999; O’Neill et al., 2004) and also as a structural component in cytoskeleton assembly in plants (Bassil et al., 2004). Recent studies have shown the presence of several genes associated with boron transport and tolerance in plants (Takano et al., 2002; Miwa et al., 2007; Sutton et al., 2007). Arabidopsis thaliana BOR1 was the first gene shown to play a role in boron tolerance and it has 6 paralogs (BOR2 to BOR7) (Takano et al., 2002). Since the identification of BOR1, similar boron transport proteins have been identified in rice (Oryza sativa), wheat, and barley (Nakagawa et al., 2007; Reid, 2007; Sutton et al., 2007). Homologs of BOR1 are found to be present in many organisms including yeast, plants, and humans (Nakagawa et al., 2007; Sutton et al., 2007; Takano et al., 2007).

High levels of boron lead to degradation of its own exporter, Bor1, in Arabidopsis thaliana (Miwa et al., 2007) but degradation of BOR1-GFP is inhibited by concamycin A, which is a specific V-ATPase inhibitor (Takano et al., 2005). Thus AtBor1 cannot be used to produce genetically modified plants that can grow where boron toxicity in soil is a problem. However, transgenic plants expressing BOR4, one of the 6 paralogs of BOR1, showed a high tolerance to toxic levels of boron (Miwa et al., 2007). Multicopies of BOT1, a BOR1 ortholog, could also provide boron-toxicity tolerance in barley (Sutton et al., 2007).

The yeast Saccharomyces cerevisiae has been used as model organism for the characterisation of plant boron tolerance genes (Nozawa et al., 2006; Takano et al., 2006; Sutton et al., 2007; Takano et al., 2007). Barley Bot1 has been shown to provide boron tolerance using yeast as a model (Sutton et al., 2007). Additionally, different Arabidopsis thaliana cDNAs that are related to boron tolerance have been monitored in yeast cells (Nozawa et al., 2006). Yeast can grow under very high concentrations of boron and is considered to be a highly boron-tolerant organism (Nozawa et al., 2006). The yeast BOR1 gene was characterized in detail. It is localised to the plasma membrane and functions as a boric acid exporter across the cell membrane. The Δbor1 (bor1 deletion mutant) yeast strain overaccumulates boron inside its cells (Takano et al., 2002). Cells that overexpress BOR1 have lower intracellular boron and show resistance to boron treatment (Nozawa et al., 2006; Jennings et al., 2007). In addition to BOR1, 2 other yeast transporter genes, DUR3 and FPS1, seem to play a role in boron tolerance, but their significance is not clear. Overexpression of FPS1 and DUR3 has controversial effects on cellular boron levels; while FPS1 expression lowers the protoplasmic boron concentrations, DUR3 expression leads to a small increase (Nozawa et al., 2006). Recently, another yeast boron transporter gene ATR1 (YML116w) has been reported to be the strongest yeast boron transporter (Kaya et al., 2009). ATR1 is localized in cell membranes and vacuoles and atr1Δ cells have been shown to be more sensitive to boric acid than previously identified yeast boron transporter mutants (Kaya et al., 2009).

V-ATPases are proton pumps located in endosomes, lysosomes, golgi, and vacuoles which maintain acidification of organelles (Nishi & Forgac, 2002). Eukaryotic V-ATPases are structurally and functionally highly conserved pumps in mammals, plants, and yeast. V-ATPase is composed of 2 domains, an integral membrane domain V0 and a membrane-bound domain V1 (Wilkens et al., 1999; Sze et al., 2002). The function of V-ATPase subunit E has not been defined in plants, but mutational
analysis from yeast suggests that the V1 and V0 sectors of the V-ATPase are assembled by subunit E.

Genetic variation for boron tolerance is expressed through different mechanisms in different barley genotypes. Sahara, one of the boron-tolerant barley genotypes, accumulates less boron in leaf blades due to a mechanism of active boron efflux from the root (Sutton et al., 2007). In contrast to Sahara, the Turkish boron-tolerant barley genotype Anadolu accumulates the same amount of boron in its leaf tissue as a boron-sensitive barley genotype, indicating it has a different mechanism responsible for boron tolerance (Torun et al., 2003).

The aim of this study was to understand the boron tolerance mechanisms in the boron-tolerant Turkish barley Anadolu cultivar. Using a proteomic approach, we demonstrated several differentially expressed proteins upregulated after boron treatment in the Anadolu genotype. One protein, vacuolar proton-translocating ATPase (V-ATPase) subunit E, is overexpressed in yeast and provides boron tolerance.

Materials and methods

Plant growth and proteomic analysis

Turkish boron-tolerant barley genotype (*Hordeum vulgare* L. cv. Anadolu) and boron-sensitive barley genotype (*Hordeum vulgare* L. cv. Hamidiye) seeds were used. Seeds were surface-sterilized with 5% sodium hypochlorite and grown in Hoagland's solution for 5 days under controlled environmental conditions (23 ± 2 °C with 16-h light/8-h dark photoperiods at a light intensity of 40 μmol m⁻² s⁻¹, and with 60%-80% relative humidity). Boric acid was applied at a final concentration of 10 mM for an additional 4 days. At the end of the growing period, the control and treated barley leaves were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until the protein and RNA extraction steps.

The protein extraction was performed with 3 g of leaf sample using the TRizol (phenol/guanidine isothiocyanate) reagent (Kirkland et al., 2006). Next, the protein pellet was vacuum-dried in a centrifuge evaporator. The resultant pellet was then dissolved in a rehydration-solubilisation buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, and 2.5% (v/v) ampholyte (pH 3-10) followed by centrifugation at 8000 × g for 10 min at 4 °C. The protein-containing supernatant was either used for 2-DE or stored in -80 °C for further use. The total protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

The protein samples (500 μg) were diluted in a final volume of 330 μL of rehydration buffer and 300 μL of each sample was applied onto IPG strips (pH 3-10 non-linear, 17 cm). Rehydration for transferring proteins onto the strips was done at 50 V for 15 h using Bio-Rad's IEF system (Bio-Rad Protean IEF Cell) with subsequent isoelectric focusing at 20 °C under mineral oil for 56 kVh (0-500 V 2000 Vh, 500 V 2000 Vh, 500-3500 V 12 kVh, 3500 V 40 kVh). Prior to SDS polyacrylamide gel electrophoresis, the strips were equilibrated in 6M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% glycerol with DTT 2% for 15 min and with iodoacetamide 2.5% for the alkylation of the reduced sulphhydryl groups for another 15 min. Electrophoresis was carried out at 220 V for 6 h (Bio-Rad Protean II xi Cell). Gels were stained with colloidal Coomassie Brilliant Blue. Three replicates of the 2-DE gels were analysed and replicate spots in 3 gels were excised and subjected to overnight in-gel digestion with trypsin. Samples were desalted using Ziptip (Millipore) before running. Finally, peptides that were isolated from the digested proteins were identified using Mass spectrometry / Mass spectrometry (MS/MS) spectra. A LTQ XL linear ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an electrospray ionisation (ESI) source, and a hybrid triple quadrupole/linear ion trap 4000 Q-TRAP instrument (Applied Biosystems/ MDS Sciex, Ontario, Canada), equipped with a turboion spray source, were used for the MS/MS study. The Mascot search engine (Matrix Science, London, UK) with the NCBI nr protein database (National Center for Biotechnology Information, Bethesda, MD, USA) were used to sequence protein spots.

Gene expression analysis

To both the Anadolu and Hamidiye cultivars 10 mM of boric acid stress was applied for 4 days as described in the proteomic experiments. Total RNA from the samples was isolated using the RNeasy Mini Kit (Qiagen). Traces of genomic DNA were removed by DNase treatment (DNase RQ1, Promega). cDNA
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synthesis was performed using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The cDNA was used as template to amplify a 159 bp fragment for V-ATPase subunit E gene and a 524 bp fragment for the internal control barley GPD gene. Maxima™ SYBR Green qPCR Master Mix was used (Fermentas, Germany) to perform the Quantitative Real Time PCR (Q-RT-PCR) assays. Three independent experiments were performed for the Q-RT-PCR assays with the Bio-Rad iQ5 Real-Time PCR system. The amounts of RNA in each reaction normalised the barley GPD gene. Q-RT-PCR data analysis was performed with Bio-Rad iQ5 software using Pfaffl’s model (Pfaffl 2001). Primers used for the amplification of HvATPase subunit E (accession number U84268) were V ATPRTF-TATCAAGTGCTTCAGCCAGGA and V ATPRTR – AGTACAGCTGGCTCTTTCAACGA, and for the amplification of the barley GPD gene were HGPDRTF-TGAGGGTTTGATGACC ACTGTCCA and HGPDRTF-CCGTTAAGTTCTGGGAGCACC TT. The conditions for PCR amplification were as follows: 95 °C for 5 min, 40 cycles at 94 °C for 25 s, 60 °C for 25 s, and 72 °C for 30 s.

Overexpression of the barley V-ATPase subunit E gene in yeast

The V-ATPase subunit E gene was amplified by Pfu polymerase (Fermentas) from barley cDNA using primers VATPEF-AGGATCCATGAACGACACCGATGTCTCAGGC and VATPER-ACTCGAGTTATGCCACACAAGAGCTTTCGG containing BamHI and XhoI restriction sites respectively. The amplified fragment was first cloned into the Sma1 site of pBS II KS (Stratagene) and then moved into BamHI/XhoI sites of yeast respectively. The amplified fragment was first cloned into the Smal1 site of pBS II KS (Stratagene) and then moved into BamHI/XhoI sites of yeast high copy expression vector p426GPD to make p426- V-ATPase subE. We used Saccharomyces cerevisiae strains with a BY4741 background (MATa his3 leu2 met15 ura3). YPD media (2% glucose, 2% peptone, 1% yeast extract, and 2% agar for solid media) or YNB media supplemented with required amino acids or bases were used to grow the cells. For the solid media growth assays, wild type yeast cells were transformed with either empty vector p426GPD or V-ATPase subunit E cDNA containing p426GPD over-expression vector, using the lithium acetate (LiAc) method (Gietz & Schiestl, 1995). Yeast cells (shaken at 200 rpm, at 30 °C) that had been incubated overnight were diluted to OD600 = 0.2 with distilled water and 5 μL of these cultures were transferred to spots on solid YNB (~ura; 2% glucose at pH = 6) plates without boric acid or with 90 mM boric acid. The cells were incubated for 5 days at 30 °C and the plates were photographed.

Results

A proteomic approach was used to identify proteins that were overexpressed by boron stress in the Anadolu barley cultivar, which is known to be tolerant of high levels of boron in soil. Mass spectrometry analysis and database searching helped to identify 9 spots representing 7 different proteins (3 spots were identified as the same protein). The 7 identified proteins are namely, ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain), a thaumatin-like protein TLP5, a basic pathogenesis related protein PR5, a RNase S-like protein, vacuolar proton-translocating ATPase subunit E, a PSI type III chlorophyll a/b-binding protein, and a light-harvesting complex I LHC I (Figures 1-2, Table). From among these proteins, Hordeum vulgare V-ATPase subunit E was identified as a boron-responsive protein by MS/MS spectra (with 38% sequence coverage, MW 26.2kDa / pI 6.57, accession number 4099148).

Since the V-ATPase subunit E protein was identified as one of the boron-regulated proteins using mass spectrometry, further experiments were carried out to see whether V-ATPase subunit E mRNA levels are regulated by boron treatment. The amount of V-ATPase subunit E mRNA in leaves dramatically decreased by 0.6-fold after 1 day of boron treatment and continued to decrease by approximately 0.8-fold for the rest of the treatment period (Figure 3). In the roots of samples treated with boron, V-ATPase subunit E mRNA levels showed a dynamic regulation with 0.4-fold upregulation on the 1st and 4th days and 0.2-fold downregulation on the 2nd day.

Since Anadolu is a boron-tolerant cultivar of Turkish barley, we wanted to compare the expression levels of V-ATPase subunit E between it and the boron-sensitive barley cultivar Hamidiye under boron stress. After 10 mM of boron treatment for 4
days, V-ATPase subunit E mRNA levels decreased in both Anadolu and Hamidiye leaves (Figure 4). However, when we compared the native expression level of V-ATPase subunit E in control samples, the Anadolu control sample showed a 6-fold higher level than that of the Hamidiye control. Constitutively high expressions of subunit E in Anadolu barley might play a role in its boron tolerance.

The yeast *Saccharomyces cerevisiae* has been used extensively to elucidate plant boron tolerance genes. As seen in Figure 5, yeast cells carrying the empty vector (p426GPD) were not able to grow in the presence of 90 mM boron, but cells containing p426GPD-V-ATPase subunit E provided boron resistance and were able to grow in 90 mM boron which is a toxic level for yeast. Thus, the V-ATPase subunit E cDNA of barley might play a role in boron tolerance.

**Discussion**

After stress conditions, plants can change their gene expression and protein accumulation, causing the reduction of normal growth and development and loss of leaf area due to inhibition of photosynthesis from a decrease of chlorophyll concentrations and CO₂ fixation (Terzi et al., 2010; Yang et al., 2010). After toxic boron concentration in the Anadolu barley genotype, this observation was confirmed by identification of 3 proteins that are related to the photosynthetic mechanism. These proteins are ribulose 1,5-bisphosphate carboxylase/oxygenase large chain (RuBisCo large chain), a PSI type III chlorophyll a/b-binding protein, and a light-harvesting complex ILHC I (spot 1, spot 8, and spot 9, respectively). On the other hand, the thaumatin-like protein TLP5 (spot 2) increased its protein level. Öz et al. (2009) also reported an increased mRNA level TLP5 gene in barley. The basic pathogenesis related protein PR5 (spot 3), and the RNAse S-like protein (spots 4, 5, and 6) are environmental stress-related proteins that are expressed in plant leaves under several stress treatments (Shimizu et al., 2001; 2002; Sarowar et al., 2005; Patterson et al., 2007).

Plant V-ATPase is a primary-active proton pump located in the vacuolar membrane (tonoplast) and in various other components of the endomembrane system of the plant cell (Li & Zhang, 2004). Plant V-ATPase levels, subunit composition, and subunit stoichiometry vary in different types of tissues,
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environmental stresses, and developmental states of the plants (Luttge & Ratajczak, 1997; Ratajczak, 2000; Kaya et al., 2009). The isoforms of V-ATPase subunit E have been detected in several plant species (Kawamura et al., 2000; Kluge et al., 2003). It has also been stated that accumulation of subunit E is slightly modified by salt stress in barley (Dietz et al., 1995; Dietz & Arbinger, 1996).

Figure 2. Segments of 2D gel showing upregulation of the proteins.
## Table

List of proteins which are differentially upregulated after boron treatment in Anadolu barley leaves.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein identity</th>
<th>Accession number</th>
<th>Reference organism</th>
<th>Matched peptide</th>
<th>Sequence coverage (%)</th>
<th>MOWSE score</th>
<th>Theoretical MW (kDa) / pI</th>
<th>Experimental MW (kDa) / pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ribulose 1,5-bisphosphate carboxylase/oxygenase large chain</td>
<td>14017580</td>
<td>Triticum aestivum</td>
<td>AGVGFKAGVKLTYYTPEYETKDTDIILAARREMTHGFDLLRFEEFEFVDTITDK</td>
<td>10</td>
<td>251</td>
<td>52.8/6.22</td>
<td>55.0/7.20</td>
</tr>
<tr>
<td>2</td>
<td>thaumatin-like protein; TLP5</td>
<td>56682582</td>
<td>Hordeum vulgare</td>
<td>LDPGQSWQLNPMTAGARVSQEGPATLAETYTLGQGGNREMTLGFVDLLRFEEFEFVDTITDK</td>
<td>16</td>
<td>103</td>
<td>24.9/6.04</td>
<td>24.0/7.00</td>
</tr>
<tr>
<td>3</td>
<td>basic pathogenesis-related protein; PR5</td>
<td>2344818</td>
<td>Hordeum vulgare</td>
<td>VSGQQPTLAETYTLGQGANK</td>
<td>8</td>
<td>64</td>
<td>25.2/6.54</td>
<td>20.0/7.10</td>
</tr>
<tr>
<td>4</td>
<td>RNase S-like protein</td>
<td>21954110</td>
<td>Hordeum vulgare</td>
<td>LYNTEHIK</td>
<td>3</td>
<td>50</td>
<td>27.6/6.58</td>
<td>26.0/6.70</td>
</tr>
<tr>
<td>5</td>
<td>RNase S-like protein</td>
<td>21954110</td>
<td>Hordeum vulgare</td>
<td>ADVLGALAEQGINPDYRLYNTEHIK</td>
<td>10</td>
<td>139</td>
<td>27.6/6.58</td>
<td>27.0/6.80</td>
</tr>
<tr>
<td>6</td>
<td>RNase S-like protein</td>
<td>21954110</td>
<td>Hordeum vulgare</td>
<td>ADVLGALAEQGINPDYRLYNTEHIK</td>
<td>10</td>
<td>107</td>
<td>27.6/6.58</td>
<td>27.5/6.90</td>
</tr>
<tr>
<td>7</td>
<td>vacuolar proton translocating ATPase (V-ATPase) Subunit E</td>
<td>4099148</td>
<td>Hordeum vulgare</td>
<td>QIQQMV'RAGEISVSAAEFNIEKLQIVEAEEKIEYSMQNLASRLQAQQDDLVNKLTVQGELRLKEPAVLLRIVFNTVDAVRLVAA</td>
<td>38</td>
<td>486</td>
<td>26.2/6.57</td>
<td>30.0/7.00</td>
</tr>
<tr>
<td>8</td>
<td>PSI type III chlorophyll a/b binding protein</td>
<td>430947</td>
<td>Arabidopsis thaliana</td>
<td>LQDWYNPGSMGKQYFLGEK</td>
<td>7</td>
<td>65</td>
<td>29.1/8.61</td>
<td>23.0/6.20</td>
</tr>
<tr>
<td>9</td>
<td>light-harvesting complex E; LHC1</td>
<td>544700</td>
<td>Hordeum vulgare</td>
<td>KYPGAFDPLGSKNIYGP</td>
<td>8</td>
<td>84</td>
<td>24.2/8.11</td>
<td>21.0/6.00</td>
</tr>
</tbody>
</table>
Proteomic changes during boron tolerance in barley (Hordeum vulgare) and the role of vacuolar proton-translocating ATPase subunit E

Our results show that protein levels of V-ATPase subE are upregulated under boron stress in barley. To see expression of mRNA levels of V-ATPase subE, we performed Q-RT-PCR analyses that showed that the V-ATPase subunit E gene is downregulated by boron, suggesting a possible post-translational regulation. Hanitzsch et al. (2007) studied regulation of V-ATPase subunit isoforms in different organs of Arabidopsis. They reported that Western blot analysis of VHA-E isoforms showed transcript abundances not directly translated into protein abundances, suggesting translational and post-translational regulation that differs between roots and shoots. In addition, Kabała and Kłobus (2008) studied the activity of vacuolar proton transporting ATPase (V-ATPase) and vacuolar proton transporting pyrophosphatase (V-PPase) under salt stress in cucumber roots. They demonstrated that changes in enzyme activities of V-ATPase (CsVHA-A), subunit C of V-ATPase (CsVHA-c), and V-PPase (CsVP) were not correlated with mRNA expression levels of these genes after salt treatment. Such results suggest that alterations of proton pump activities under salinity are instead due to the post-translational alterations.

Figure 3. Expression levels of V-ATPase subunit E in Anadolu barley roots and leaves. A- mRNA levels of V-ATPase subunit E in the leaf cells after 10 mM boron treatment for 1-4 days, including a no boron treatment control (indicated as C). B- mRNA levels of V-ATPase subunit E in the Anadolu barley root cells after 10 mM boron treatment for 1-4 days, including a no boron treatment control (indicated as C). Data are the average of 3 biological replicates. Error bars represent standard errors.

Figure 4. Expression levels of V-ATPase subunit E in Anadolu and Hamidiye barley leaves after 4 days of 10 mM boron treatment. Data are the average of 3 biological replicates. Error bars represent standard errors.

Figure 5. Boric acid tolerance of yeast cells with empty vector (p426GPD) and with V-ATPase subunit E-p426GPD. YNB plates containing no boric acid (as control) and YNB plates containing 90 mM boric acid (as stress). Plates were incubated at 30 °C for 5 days.
induced by salt. As concluded from these studies, mRNA levels of V-ATPases do not directly correlate with their protein levels under stress conditions and our expression analyses for V-ATPase subunit E are consistent with these studies.

The relationship between boron and plant cell walls has been known for several years. When boron is limited, the stability and functioning of plant cell membranes are affected (Bolaños et al., 2004; Camacho-Cristóbal et al., 2008). One of the known physiological roles of boron in plants is cross-linking of the pectin rhamnogalacturonan-II in the cell wall (Kobayashi et al., 1996; Ishii et al., 1999; O’Neill et al., 2004; Wimmer et al., 2009). Wimmer et al. (2009) isolated membrane-associated boron-interacting proteins by boronate affinity chromatography in the roots of Arabidopsis thaliana and Zea mays. One of the boron-interacting proteins they found was a V-type ATPase, a similar protein to one that was also isolated in this work.

Boron tolerance shows through different mechanisms in barley genotypes. Sahara, a boron-tolerant barley genotype, accumulates less boron in leaf blades due to a mechanism of active boron efflux from the root (Sutton et al., 2007). However, the Turkish boron-tolerant barley genotype Anadolu accumulates the same amount of boron in leaf tissues as the boron-sensitive barley genotype Hamidiye, indicating a possible internal tolerance mechanism such as complexation of boron in cell walls or compartmentation of boron at the cellular or organ levels (Torun et al., 2003). Our analyses also showed that mRNA levels of V-ATPase subE in Anadolu barley are 6-fold higher under normal conditions than in Hamidiye. The possible role of V-ATPase subE in boron tolerance needs to be investigated further in different plant species.

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References


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