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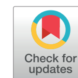
Transcriptomic analysis of boron hyperaccumulation mechanisms in *Puccinellia distans*



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HIGHLIGHTS

- *Puccinellia distans* accumulates extremely high levels of soil boron.
- Hyperaccumulation is accompanied by many transcriptomic changes.
- Changes are seen in the malate pathway and cell wall components.
- Putative boron transporters and aquaporins are up-regulated.

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ABSTRACT

Puccinellia distans, common alkali grass, is found throughout the world and can survive in soils with boron concentrations that are lethal for other plant species. Indeed, *P. distans* accumulates very high levels of this element. Despite these interesting features, very little research has been performed to elucidate the boron tolerance mechanism in this species. In this study, *P. distans* samples were treated for three weeks with normal (0.5 mg L^{-1}) and elevated (500 mg L^{-1}) boron levels in hydroponic solution. Expressed sequence tags (ESTs) derived from shoot tissue were analyzed by RNA sequencing to identify genes up and down-regulated under boron stress. In this way, 3312 differentially expressed transcripts were detected, 67.7% of which were up-regulated and 32.3% of which were down-regulated in boron-treated plants. To partially confirm the RNA sequencing results, 32 randomly selected transcripts were analyzed for their expression levels in boron-treated plants. The results agreed with the expected direction of change (up or down-regulation). A total of 1652 transcripts had homologs in *A. thaliana* and/or *O. sativa* and mapped to 1107 different proteins. Functional annotation of these proteins indicated that the boron tolerance and hyperaccumulation mechanisms of *P. distans* involve many transcriptomic changes including: alterations in the malate pathway, changes in cell wall components that may allow sequestration of excess boron without toxic effects, and increased expression of at least one putative boron transporter and two putative aquaporins. Elucidation of the boron accumulation mechanism is important in developing approaches for bioremediation of boron contaminated soils.

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1. Introduction

Boron (B) is a micronutrient with roles in many physiological and biochemical pathways in plants: the metabolism of nucleic acids, carbohydrates, and proteins, cell wall synthesis and structure (Matoh, 2001) and membrane integrity and function (Goldbach, 1997; Goldbach et al., 2001; Marschner and Marschner, 2012). For example, boron provides stability to the cell wall by crosslinking rhamnogalacturonan-II, a pectic polysaccharide found in the primary wall. The majority of soil boron is found in Turkey (72.5% of world reserves), followed by Russia, USA, and China (BOREN, 2012). Boron levels are affected by rainfall and boron-rich groundwater can cause toxicity in arid and semi-arid regions of the world (Nable et al., 1997; Tanaka and Fujiwara, 2008; Sang et al., 2015). Boron deficiency and toxicity are observed within a very narrow concentration range. Soil concentrations less than 0.5 mg kg^{-1} are

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associated with deficiency while levels greater than 5.0 mg kg^{-1} cause toxicity (Ryan and Rashid, 2006). Toxicity alters cell division and reduces cell growth thereby causing decreased crop growth and productivity (Yau et al., 1995; Ozturk et al., 2010). Typical symptoms of boron toxicity are chlorosis and/or necrosis of mature leaf margins and increased cell wall rigidity (Ozturk et al., 2010).

Some plant species tolerate high boron concentrations. One such species, *Puccinellia distans* (weeping alkali grass) was found to be extremely tolerant of excess boron (Padmanabhan et al., 2012). Under hydroponic conditions, *P. distans* shoots accumulated approximately 6000 mg kg^{-1} (Bar, 2015). Other work suggested that a related species, *Puccinellia frigida*, is also highly tolerant to boron with approximately 4000 mg kg^{-1} accumulation in shoots (Rámila et al., 2015, 2016). Puccinellia species are thus considered important candidates for phytoremediation applications. Compared to chemical, physical and biological boron-removal technologies, phytoremediation and phytoextraction are clean, simple, cost-effective, environmentally friendly methods for removing toxic elements from soil (Padmavathiamma and Li, 2007; Ali et al., 2013; Rámila et al., 2015).

The first boron transport mechanism described in plants was passive transport of uncharged boric acid across the plasma membrane (Marschner and Marschner, 2012). Early studies in bread wheat determined that the *Bo1*, *Bo2*, and *Bo3* nuclear genes control boron tolerance (Paull et al., 1992). The *BoT1* and *BoT2* genes were then identified in barley (Jenkin, 1993) and durum wheat (Jamjod, 1996). Later studies indicated that these genes encode active boron transporter proteins and that boron uptake was hindered by mutations in BOR transporter genes and metabolic inhibitors in various species (Takano et al., 2001; Uraguchi and Fujiwara, 2011). Major intrinsic channel-like transporter proteins (MIPs) also play a role in boron uptake (Dannel et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2007). Indeed, two aquaporins in *A. thaliana* were shown to have boric acid channel activity (Takano et al., 2006, 2008).

The main goal of this study was to examine the boron hyper-accumulation/tolerance mechanisms of *P. distans* using an RNA sequencing approach. RNA sequencing was employed because transcriptome data provide genome, whole transcriptome and gene level comparisons between treatments as has been shown in other hyperaccumulator plants (Blande et al., 2017; Chen et al., 2017; Yang et al., 2017). *P. distans* plants were treated with normal (0.5 mg L^{-1}) and elevated (500 mg L^{-1}) boron levels in hydroponic solution and shoot tissues were used for RNA sequencing to identify differentially expressed transcripts. Differentially expressed transcripts were then annotated at the protein level and gene ontology and pathway analyses were performed against *O. sativa* and *A. thaliana* to identify the up and down-regulated genes and proteins that may play roles in *P. distans* boron tolerance and/or hyperaccumulation.

2. Methods

2.1. Plant material and boron treatment

Seeds of *P. distans* collected from a boron mining site in Kirka-Eskisehir, Turkey (39° 17′ 23.7156″ and 30° 31′ 33.4812″) (Babaoğlu et al., 2004), were germinated in potting soil. The germinated seedlings were grown for 4 weeks in a growth chamber maintained at 25 ± 2 °C, 60% relative humidity and 16 h photoperiod (Stiles et al., 2010). Plants were then transferred to half strength Hoagland solution (Hoagland and Arnon, 1950). After a week of growth under hydroponic conditions, plants were separated into two treatment groups with three replicates each: 0.5 mg L^{-1} boron was applied to the control group and 500 mg L^{-1} was applied to the stress group (Stiles et al., 2010). The boron concentration in the Hoagland solution was adjusted with boric acid and the solution was changed once every three days for three weeks. After 3 weeks of treatment, plants were removed from the Hoagland solution and rinsed with RNase-free water to remove any contamination. The shoot tissues were then frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA isolation

Total RNA was isolated from the control and boron stressed shoot samples using an RNeasy Plant Mini Kit (Qiagen, Maryland, USA). The quality and quantity of isolated shoot RNA from control and stress samples were measured using a Nanodrop ND-100 device (Nanodrop Technologies, Wilmington, DE, USA).

2.3. RNA sequencing and analysis

Total RNA samples were processed using a TruSeqTM RNA Sample Preparation Kit (Illumina, Tokyo, Japan) for cDNA library construction and subsequent EST identification. ESTs were sequenced for control and stress libraries using an Illumina HighSeq 2000 platform (Takara, Tokyo, Japan) to generate 125 bp paired end (PE) reads. RNA sequencing was performed by GATC Biotech (Constance, Germany). Raw data consisted of 45 million reads for each sample with read length fixed at 125 nucleotides.

The Cutadapt2 (version 1.9.1) program (Martin, 2011) was employed with default parameters to remove adapter sequences and low quality nucleotides from raw reads. Reference transcriptome construction was performed using the Trinity (version 2.2.0) assembly tool (PMID: 23845962) (Haas et al., 2013). All reads from the control and stress samples were treated the same. The reference transcriptome was used to map the cleaned reads from control and stress datasets individually using the Bowtie2 program (version 2.1.0) (Langmead and Salzberg, 2012). Mapping information for each dataset was saved in a binary alignment information (bam) file. The bam files were used as inputs for differential gene expression analysis performed with the Cufflinks (version 2.2.1) pipeline (Cufflinks, Cuffmerge, and Cuffdiff) (Trapnell et al., 2012). Cuffdiff selects differentially expressed transcripts based on a *t*-test comparing the normalized counts for each treatment and generates a p-value for each comparison. Results were analyzed and visualized using the cummeRbund (version 2.15) R statistics package (Goff et al., 2013). Differentially expressed gene sequences were selected based on q-value (q-value threshold < 0.05, where q-value is the false discovery rate adjusted p-value of the test statistic). Differentially expressed candidates were then annotated using Blast2GO (version 4.0.7) (Conesa et al., 2005) against a custom protein database which included *Oryza sativa* and *Arabidopsis thaliana* proteins in UniProt Knowledgebase (release date: November 2016). Gene ontology analysis was carried out with QuickGO (Binns et al., 2009). Protein functional classification based on gene ontology was performed by PANTHER Protein Classification System (Accession date: November 2016) (Mi et al., 2017). Annotated transcripts were searched against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Tanabe and Kanehisa, 2012) to reveal the pathways in which up and down-regulated transcripts have roles.

2.4. Evolutionary conservation

Phylogenetic analyses of boron transporters and other ion transporters of multiple species were performed to determine their sequence similarity and evolutionary conservation. This analysis included all of the fourteen transporters found to be up or

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