ELUCIDATION OF BORON TOLERANCE MECHANISMS IN *Puccinellia distans (Jacq.) Parl.* USING A TRANSCRIPTOMIC APPROACH

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Saniye Elvan ÖZTÜRK

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We approve the thesis of **Saniye Elvan ÖZTÜRK**

Examining Committee Members

_____

Prof. Dr. Anne FRARY
Department of Molecular Biology and Genetics, Izmir Institute of Technology

_____

Prof. Dr. Talat YALÇIN
Department of Chemistry, Izmir Institute of Technology

_____

Assoc. Prof. Dr. Hüseyin Çağlar KARAKAYA
Department of Molecular Biology and Genetics, Izmir Institute of Technology

_____

Prof. Dr. Hülya İLBİ
Department of Horticultural, Ege University

_____

Assist. Prof. Dr. Seçkin EROĞLU
Department of Genetics and Bioengineering, Izmir University of Economics

6 June 2017

_____

Prof. Dr. Anne FRARY
Supervisor, Department of Molecular Biology and Genetics,
Izmir Institute of Technology

_____

Prof. Dr. Volkan SEYRANTEPE
Head of Department of Molecular
Biology and Genetics

_____

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

ELUCIDATION OF BORON TOLERANCE MECHANISMS IN *Puccinellia distans* (Jacq.) Parl. USING A TRANSCRIPTOMIC APPROACH

The amount of boron in soil is important for agronomic plants. As an abiotic stress condition, boron toxicity causes significant decreases in crop yields. *Puccinellia distans* (Jacq.) Parl. (*P. 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 analyzed by RNA sequencing to identify genes and miRNAs related to boron tolerance and hyperaccumulation. The results indicated that the hyperaccumulation mechanism of *P. distans* involves 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. MiRNAs are also altered under stress conditions. The presence of miRNAs as stress regulator elements is an example of post-transcriptional regulation of stress related mechanisms. Additionally these small RNAs could affect their target genes by positive or negative regulation. Therefore, changes not only in miRNAs but also in their targets are important to understand their roles in hyperaccumulation. For example, down-regulation of miRNA under stress could cause target accumulation. These mechanisms could be key in plant adaptation to new conditions. Elucidation of the boron accumulation mechanism is important in developing approaches for bioremediation of boron contaminated soils.

ÖZET

Puccinellia distans (Jacq.) Parl.'DA BOR TOLERANS MEKANİZMALARININ TRANSKRİPTOMİK YAKLAŞIM KULLANILARAK ANLAŞILMASI

Topraktaki bor miktarı tarımı yapılan bitkiler için önemlidir. Abiyotik stres durumu oluşturması nedeniyle bor toksisitesi ürün verimliliğini önemli ölçüde azaltır. *Puccinellia distans* (Jacq.) Parl. (*P. distans*), çorak çimi, tüm dünyada yaygın olarak bulunur ve diğer bitki türleri için öldürücü olabilen dozlarda bor barındıran topraklarda yaşayabilir. Hatta *P. distans,* bu elementi çok yüksek oranda biriktirme özelliğine sahiptir. Bu kadar ilginç özelliklerine rağmen, *P. distans'*daki bor tolerans mekanizmalarının aydınlatılmasına yönelik yapılmış çalışma çok azdır. Bu çalışmada, *P. distans* bor toleransı ve hiperakümülasyonuyla ilişkili genler ve miRNA'ların RNA dizileme yöntemiyle tanımlanmasına yönelik analizler yapılmıştır. Sonuçlar göstermiştir ki, *P. distans*'daki hiperakümülasyon mekanizması malat yolağında değişimlere, borun toksik etki göstermeden biriktirmesine izin veren hücre duvarı değişimlerine ve en az bir bor transport proteini ile iki potansiyel aquaporinin yer aldığı pek çok transkriptomik değişime neden olmuştur. Stres koşullarında miRNA'larda da değişimler olmaktadır. MiRNA'ların stres düzenleyici element olarak bulunmaları, stresle ilişkili mekanizmalardaki post-transkripsiyonel düzenlemeye bir örnektir. Ayrıca bu küçük RNA'lar hedef genlerini de pozitif ya da negatif düzenlemeyle etkileyebilirler. Bu nedenle bu RNA'ların hiperakümülasyondaki rollerini anlamak için sadece miRNA değişimlerinin değil, onların hedef genlerindeki değişimlerin de anlaşılması önemlidir. Örneğin, bir miRNA'nın stres altında miktarındaki azalma, onun hedef geninin miktarında artışa neden olabilir. Bu mekanizmalar, bitkilerin yeni bir ortama alışmasındaki/uyum sağlamasındaki anahtar mekanizma olabilirler. Bor biriktirme özelliğinin aydınlatılması, bor bakımından kirlenmiş toprakların arındırılması (biyoremediyasyon) için yaklaşım geliştirmede önemlidir.

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CHAPTER 1

INTRODUCTION

1.1. Boron

Boron is a member of subgroup III of metalloids [1] and has properties between metal and non-metal elements. It has only three valence electrons, thus it is a quite small molecule. Despite its low cosmic abundance, B distribution ranges widely on terrestrial Earth (5 mg/kg in basalts; 100 mg/kg in shales) and in the ocean (~4.5 mg l^{-1}) [2-3]. The majority of soil boron is found in Turkey (72.5 % of world reserves), followed by Russia, USA, and China [4]. Important boron reserves are located in the northwest part of Turkey in Balikesir, Bursa, Kutahya and Eskisehir provinces [5].

In soil, the present structure of boron is as boric acid [3]. Therefore, boric acid easily leaches from soil under high rainfall conditions. Thus boron levels decrease and this causes boron deficiency [3, 6]. Contrarily, low rainfall causes build-up of boron in the soil and toxicity [7]. Therefore, the availability of boron in soil and irrigation water is important for agriculture [8]. Because boron levels are affected by rainfall and boron-rich groundwater, toxicity is observed in arid and semi-arid regions of the eastern Mediterranean, western Asia/north Africa, the Indian subcontinent, China, Japan and South America [8-10].

Thus, soil boron concentrations vary from deficient to toxic in different regions of the world [11]. The levels of boron that cause deficiency and toxicity vary among plants. However, in general, the difference between boron deficiency and toxicity is observed within a very narrow range. Soil boron concentrations less than 0.5 mg kg^{-1} are associated with deficiency while levels greater than 5.0 mg kg^{-1} cause toxicity [12]. The tipping point between deficiency and toxicity is even finer when internal boron concentration is considered. For example, soybean leaf elongation was optimal at 10 to 12 mg kg^{-1} dry weight and impaired at both higher and lower concentrations [13].

1.1.1. Boron in Plants

The first demonstration of boron requirements of plants was in the early 1920s [14], and it is now known to be a micronutrient for plant development [15]. After absorption of boron by the root cells, this micronutrient is loaded into xylem by a passive mechanism which involves boron diffusion across the lipid bilayer of cells. A second transport system is facilitated transport of boron from the membrane by boric acid channels via MIPs (major intrinsic proteins) [16]. Xylem loading and subsequent boron transport to shoot tissue is achieved by transpiration [17-18], however this pathway is not sufficient for boron to reach both reproductive and vegetative tissues. Thus phloem loading is needed for boron transportation [19] via boron-diol complexes [20-21]. The ability of boron to make complexes with *cis*-hydroxyl groups of sugar alcohols, such as sorbitol, facilitates its transport via the phloem. The energy dependent translocation of boron with BOR transporters was first described in *Arabidopsis thaliana* [22].

The roles of boron are in mainly three areas: organization of cell walls, membrane function and metabolic activities [23]. Most biological fluids include boron, primarily as boric acid, $B(OH)_3$, plus a small amount of borate anion $B(OH)_4$. Both boric acid and borate anion have the tendency to form complexes with a variety of sugars and other compounds containing *cis*-hydroxyl groups [24]. The main function of boron in plant cells is its ability to form borate ester complexes with apiose residues of rhamnogalacturonan II (RG-II) in pectin [25]. Therefore, boron plays a central role for cell wall structure and function. The essentiality of boron for cell wall structure was analyzed in *A. thaliana* mutants, *mur1-1* and *mur1-2*. These plants have a reduced amount of RG-II – borate complex and normal growth of the plant is affected [26].

Boron deficiency has damaging effects on many biological processes such as carbohydrate metabolism, legume nitrogen fixation [27], plasma membrane structure and cell wall integrity [28]. It was reported that under boron deficient conditions, cell wall-modifying enzymes were downregulated, thus the cell wall is loosened [28]. Boron is also a positive regulator of plasma membrane potential [29]. Therefore boron deficient conditions reduced the proton pumping activity of membranes. Boron deficiency studies make clear that boron is an important element to increase the strength of the cell and cell wall by contributing to cell wall assembly with *cis-diol* interactions, but it also has another role to regulate expression of genes involved in membrane structure and function.

Worldwide, boron toxicity is a significant problem which causes reduced agricultural yield. Boron toxicity is generally found together with alkaline and saline soils because of low rainfall conditions [28]. Boron toxicity causes decreases in crop growth and productivity [5, 11]. Boron toxicity disrupts metabolism by altering cell division and development as well as reducing cell growth. Symptoms of boron toxicity are typically observed on mature leaf margins which become chlorotic and/or necrotic [5]. The major toxicity effects of boron occur as a result of *cis-diol* interactions. These interactions have three mechanisms: *i)* boron directly interacts with cell wall structure, *ii)* boron causes metabolic disruption by binding to the ribose moieties of molecules such as adenosine tri-phosphate (ATP), and *iii)* boron disrupts cell division and development by binding ribose in free sugars and RNA [30-31].

1.2. Phytoremediation

Boron toxicity limits agricultural productivity by reducing crop yields [32] and the variety of species that can be cultivated in the contaminated region [33]. Boron cannot be easily purified from the soil, because of its interactions with soil surfaces [33]. The most common technique to remove excess boron is leaching [34]. However, this technique has some limitations, such as: *i)* it requires a high amount of water, *ii)* washing of soil causes loss of nutrients, *iii)* soils must have good drainage capacity and *iv)* removing of absorbed boron is not successful [33]. Another application for boron removal is liming [35]. The principle of this technique is changing the pH of the soil which affects boron absorption. On the other hand, this is a short term solution and also not useful for salt-rich soils. Soil replacement with low-boron soils is another choice to overcome boron toxicity, but this treatment is also expensive and subsurface boron can migrate to upper regions of the soil and recharge it. Therefore boron-removal techniques are impractical and expensive. Because of this, two new strategies are being explored: enhanced plant tolerance for boron and/or use plants for phytoremediation.

Phytoremediation is a new technology for purification of excess boron from the soil. The term phytoremediation means the use of a plant to remove or reduce toxic compounds from the soil and/or environment [33]. This new technique has several advantages: it is passive, solar-driven, less expensive, and environmentally friendly [36]. Three phytoremediation strategies have been studied: phytoextraction, phytomanagement

and phytorestoration [33]. Phytoextraction involves planting tolerant plant species which translocate contaminants to the plant's upper parts [37]. This technique is the most widely used phytoremediation strategy and hyperaccumulator plant species are often used for it. Phytomanagement is used to reduce the environmental risk posed by contaminated sites and ideal candidate plants for this purpose are poplars (Populus sp.). Phytomanagement requires perennial, fast-growing, extensive and deeply rooted plants [38]. Additionally, these plants should be tolerant for different metal and/or metalloids. Phytorestoration of soil consists of restoring a polluted place by revegetating in with a tolerant plant species [33]. This strategy was used at a US boron mine with the boron hyperaccumulator plant *Puccinellia distans* [39]. According to this strategy, such plants are used for initial vegetation and restore soil properties, and thus allowing vegetation with native species which are not tolerant to boron.

1.3. Puccinellia distans

Puccinellia distans is monocotyledonous, also known as weeping alkali grass and belongs to the family Gramineae. Therefore it has a fairly close genetic relationship with *Oryza sativa* and *Hordeum vulgare* [40-41]. While its relatives are sensitive to high boron, *P. distans* has an extraordinary capacity to tolerate this element (approximately 6000 mg B kg^{-1} under hydroponic conditions) [42-43].

Recent work by Ramila et al. (2015) suggested that a related species, *Puccinellia frigida*, is also highly tolerant to boron toxicity with approximately 4000 mg kg^{-1} accumulation in shoots [44-45]. As a result, Puccinellia species are considered to be important candidates for phytoremediation applications [46]. Phytoremediation is a more practical and promising solution for reducing the boron content of arid and semi-arid soils compared to chemical, physical and biological boron-removal technologies which are expensive and impractical [33, 47]. Despite its potential for phytoremediation, the boron hyperaccumulation mechanism(s) of Puccinellia has not yet been elucidated. Additionally it is good model to understand tolerance/hyperaccumulation mechanisms and possibly transfer them to other economically important plants.

1.4. Genetic Control of Boron Transport and Hyperaccumulation

Agricultural losses in barley due to boron toxicity were estimated up to 17 % [48]. Boron-tolerant cultivars of barley were identified with reduced boron uptake [49], however, breeding programs have not yet produced a practical solution for boron tolerance. Thus, hyperaccumulator plants are important to understand the mechanism of tolerance and to determine if this mechanism can be transferred to economical crops/plants to improve yield efficiency in agriculture.

The first boron transport mechanism described in plants was passive transport of uncharged boric acid across the plasma membrane [1]. To understand the genetic factors involved in boron transport, early studies investigated nuclear genes in bread wheat and determined that the *Bo1*, *Bo2*, and *Bo3* nuclear genes control boron tolerance [50]. The *BoT1* and *BoT2* genes were then identified in barley [51] and durum wheat [52]. Later studies indicated that these genes encode active boron transporter proteins. These findings were further supported by the fact that boron uptake was hindered by mutations in *BOR* transporter genes and metabolic inhibitors in species including *A. thaliana* [53], barley [49], sunflower [19], wheat [7], and rice [54]. In addition to these examples, *BotT1* was also observed in Puccinellia under excess boron level and was responsible for boron efflux [45]. The expression level of *BoT1* in Puccinellia was higher in roots than shoots [45]. The possible reason was to facilitate the efflux of borate anions from roots. Boron uptake is also mediated by major intrinsic channel-like transporter proteins (MIPs) in sunflower [55], squash [56], and Arabidopsis [57]. Among these MIPs, a number of aquaporins are reported to be permeable to physiologically important molecules such as boron, silicon, ammonia, hydrogen peroxide, and carbon dioxide [58]. Indeed, two aquaporins in *A. thaliana* were shown to have boric acid channel activity [59-60].

In *A. thaliana*, AtBOR1 was the protein found to be responsible for boron translocation from roots to shoots under boron depleted conditions [61]. Also AtBOR1 has polarity towards the stele side of cells [22]. The polarity feature of this transporter suggests that it determines the direction of boron transport under limited boron conditions [62]. On the other hand, AtBOR1 is transferred into the vacuole to decrease boron transport to shoots under sufficient boron. The possible reason for this relocation of the transporter protein is to eliminate accumulation of boron in the shoot and reproductive tissues [61, 63]. AtBOR2 has vacuolar sorting similar to AtBOR1 [64]. However, it has a different

physiological function. Mutation studies showed that AtBOR2 is responsible for transport of borate for cross-linking of RG-II under low boron conditions [62]. AtBOR4 protein is involved in tolerance to high amounts of boron as shown by overexpression studies in *A. thaliana* [65]. The polarity feature is also observed in AtBOR4, which has weak polarity towards soil [65]. Thus, the main function of this protein is to exclude excess boron from entering the cell from soil. These mechanisms show a direct relation between boron amount and boron-dependent regulation of genes with the physiological functions of boron transporters. Wakuta et al. (2015) showed the evolutionary relationships of boron transporters among different species. Therefore, different organisms share boron transporter proteins with similar functions [62].

The increase in roots' boron transporters provides a way to pump excess boron into the soil. However, the mechanism is not clear in shoots. Reid and Fitzpatrick (2009) suggested that this greater efflux capacity plays a role in protecting cellular metabolic pathways by transporting excess boron from intracellular regions into the cell wall [66].

1.5. EST and miRNA Studies under Stress Conditions

Transcriptomal regulation is important for an organism to develop adaptation to biotic and abiotic stresses. Transcriptomic studies led to identification of new genes which are involved in tolerance to stresses. High throughput technologies make it possible to study thousands of genes at the same time. Several studies have used the comparative transcriptomic approach to identify tolerance related genes under stress conditions [67-68].

1.5.1. MiRNAs

MiRNAs are a class of small non-coding RNAs and play key roles in post-transcriptional regulation of gene expression [69]. MiRNAs are transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes as precursors (pri-miRNA). Then, these precursors are folded into hairpin structures for the next processes which are cleavage by RNase III type enzymes Drosha and Dicer. Drosha cuts pri-miRNAs into ~70 nt pre-miRNAs which are transported from nucleus to cytosol. Dicer cleaves the transported pre-miRNAs into miRNA/miRNA duplexes which are ~20 nt in length. MiRNA-induced silencing complex (miRISC) is incorporated with one

strand of duplexes to inhibit translation or activate the degradation of mRNA by binding the sequences in the 3′-UTR of target mRNA [70]. The differences between plant and animal miRNAs are that while Drosha and Dicer enzymes work in animals, plants have DCL1 and HYL1 proteins for this activity. Additionally miRNA/miRNA duplexes are transported from the nucleus by the HST1 protein in plants, but pre-miRNAs are transported from the nucleus by Exportin-5 in animals [71].

The main features of miRNAs include: they are usually ~20-22 nt in length in animals [72] and ~20-24 nt in length in plants [73], miRNA precursors have well-predicted stem loop structure [72-74] and are evolutionary conserved from worm [75] to humans [76] or from monocots [77] to dicots [78]. On the other hand, only mature miRNAs show conservation in plants compared to animals [72]. For a small RNA to be considered as a miRNA, all of the aforementioned features should be observed, since other small non-coding RNAs may have only some of these characteristics. For example, tRNAs (transfer RNAs) have stem loop structure [79].

Living organisms encounter stress conditions in their lifespan and regulate their metabolism to such conditions with transcriptional, post-transcriptional, translational, and post-translational mechanisms. Small RNAs have roles in post-transcriptional gene regulation to guide gene silencing [80]. MiRNAs and their potential targets play vital roles for resistance to biotic and abiotic stresses in plants [80-81]. The first study about stress-related miRNAs in plants used computational analyses to identify miRNAs [82] that were then cloned in *A. thaliana* [83]. Computational and direct cloning strategies are two of the four miRNA identification approaches [84-85]. The others are genetic screening [86-87] and expressed sequence tags (ESTs) analysis [88]. In this study, an EST analysis approach was used to identify stress-related miRNAs in *P. distans*. The well-conserved features of several miRNAs [76, 88-91] allows them to be studied in genetically unknown organisms, such as Puccinellia. The EST approach is good for detection of conserved miRNAs among species, especially non-model organisms with limited genetic characterization.

1.6. Aim of the Study

The main goal of this study was to examine the hyperaccumulation/tolerance mechanisms of *P. distans* under high boron conditions. To achieve this goal, we used RNA sequencing to compare the expression profiles of plants grown under control and

boron levels that are normally toxic. Differentially expressed transcripts, mature miRNAs and possible miRNAs targets based on their sequence similarities were identified. Further analyses were annotation of transcripts and gene ontology and pathway analyses against the model organisms, *O. sativa* and *A. thaliana*. In this way, we identified candidates including an *A. thaliana* boron transporter, an *O. sativa* nodulin-26 like Intrinsic Protein (NIP) and other stress response-related transcripts in *P. distans*. In terms of miRNA analyses, possible Puccinellia miRNAs were identified and their source and target transcripts were analyzed to understand the logic behind post-transcriptional changes in hyperaccumulator plants. A greater understanding of the hyperaccumulation/tolerance mechanism(s) of the monocot *P. distans* will provide insight into ways of coping with boron stress toxicity in agriculturally important cereals such as rice, maize, and sorghum. Thus, while *P. distans* itself might be used to eliminate excess boron from soil, the tolerance mechanism(s) could be transferred to crop plants to confer boron tolerance and/or to develop a faster-growing, higher biomass phytoremediation system.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials and Boron Treatment

Seeds of *P. distans* collected from a boron mining site, Kirka-Eskisehir, Turkey (39° 17′ 23.7156" and 30° 31′ 33.4812") [92], were germinated in potting soil. The germinated seedlings were grown for 2 weeks in a growth chamber maintained at 25 ± 2°C, 60 % relative humidity and 16 h photoperiod [93]. After four weeks, plants were transferred to half strength Hoagland solution [94] and grown for one week. Plants were separated into two groups with three replicates: 0.5 mg L^{-1} boron was applied to the control group and 500 mg L^{-1} was applied to the stress group [93]. The boron concentration in 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 of any contamination with RNase-free water. The shoot tissues were then frozen in liquid nitrogen and stored at -80°C.

2.2. Methods

2.2.1. RNA Isolation

Total RNA was isolated from the 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).

2.2.2. RNA Sequencing and Analysis

Extracted 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 High-Seq 2000 platform (Takara, Tokyo, Japan) to generate 125 bp paired end (PE) reads. RNA sequencing was performed by GATCBiotech (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 [95] 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) [96]. 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) [97]. 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) [98]. Results were analyzed and visualized using the cummeRbund (version 2.15) R statistics package [99]. Differentially expressed gene sequences were extracted 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) [100] against a custom protein database which included *O. sativa* and *A. thaliana* proteins in UniProt Knowledgebase (release date: November 2016). Gene ontology analysis was carried out with QuickGO [101]. Protein functional classification based on gene ontology was performed by PANTHER Protein Classification System (Accession date: November 2016) [102]. Annotated transcripts were search against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [103] to reveal the pathways in which up and down-regulated transcripts have roles.

2.2.3. MiRNA Analysis

Since miRBase did not provide any *P. distans* miRNAs, miRNA sequences of available monocotyledons were used as positive data: *Aegilops tauschii* (88 precursors,

173 mature) [ASM34733v1], *Brachypodium distachyon* (317 precursors, 525 mature) [Bd21], *Elaeis guineensis* (6 precursors, 6 mature), *Festuca arundinacea* (15 precursors, 15 mature), *Hordeum vulgare* (69 precursors, 71 mature), *Oryza sativa* (592 precursors, 713 mature) [MSU7], *Sorghum bicolor* (205 precursors, 241 mature) [Sorbi1], *Saccharum officinarum* (16 precursors, 16 mature), *Saccharum* sp. (19 precursors, 20 mature), *Triticum aestivum* (116 precursors, 119 mature), *Triticum turgidum* (1 precursors, 1 mature), *Zea mays* (172 precursors, 321 mature) [RefGen_v2]. A plant specific negative dataset with 980 hairpins (http://118.178.236.158/PlantMiRNAPred/) was used for feature selection and creating models (learning).

There are hundreds of features that could be used for defining a pre-miRNA sequence/structure. In previous studies it was shown that feature selection and the total number of features used in a classification system for pre-miRNA prediction were essential parts of the analysis with significant impact on the accuracy of results [104-106]. For this analysis, 50 features that were selected based on their information gain (maximum) and computation time (minimum) were used (hpmfe_rf_I1, dc, dG, hpmfe_rf, efe, bpp, assl/sl, #A(((, lsr(%bp), mwmF, #U(((, dH, lscm, dS, lsr(%G-U), l(lsr), st(A-U), hpl, #G(((, sl, %C++%G/sl, %G++%C/sl, lsr(%U), #C++#U, #U++#C, %G/sl, %A++%G/hpl, %G++%A/hpl, dscs/hpl, lsr(%A), hpmfe_rf_I2, %G++%G/hpl, %G++%U/hpl, %U++%G/hpl, #G++#U, #U++#G, #A++#U, #U++#A, %A++%C/hpl, %C++%A/hpl, #mnn, #C(((, ir, orf/hpl, #U, #U++#U, #A++#C, #C++#A, %G-C/sl, *A(((). For more information about the features used and their calculation please see http://jlab.iyte.edu.tr/software/mirna.

Using the described datasets, a 1000 fold MCCV method was applied to train Decision Tree and Naive Bayes classifiers with 70% learning - 30% testing and 1:1 positive to negative ratios. The models with the highest accuracy scores were saved for miRNA hairpin prediction step.

The FASTA file containing 572,486 exon sequences of the Puccinellia transcriptome were fragmented to 500 nt long sequences with 250 nt overlaps. Then these fragments (1,402,834) were transcribed into RNA sequences by changing T to U (- strand) and creating the complementary strand (+). All sequences were folded into their secondary structures by using RNAfold [107-108] and hairpin structures were extracted. The hairpins were filtered based on their length value which was set as minimum 30. In the next step, models from the previous section were applied to hairpins and based on the

prediction scores of NB and DT values, an average of 0.95 was selected as a cut-off value leading to 87,026 and 85,808 candidate miRNA hairpins for NB and DT, respectively.

Mature sequences from the listed monocotyledons were used as positive data and a negative data set was created by shifting the mature sequences by half of their length in the hairpin sequences and using the new extracted sequence. Various features were calculated such as: sequence length, number of matches and mismatches in the mature sequence region, single nucleotide counts (4), dinucleotide counts (16), trinucleotide counts (64), distances of start and end positions to 3', 5' loop start, and loop end. These data sets were applied to Random Forest learner through 1000 MCCV, 70% to 30% learning/testing ratio. The models with the highest accuracy score (0.844) was applied to the predicted miRNA hairpins after fragmenting them to 24 nt long sequences with 6 nt overlaps. Mature candidates with minimum 15 nt long sequences and prediction score of minimum 1.0 were used for further analysis.

To analyze whether predicted mature sequences were similar to known plant miRNA mature sequences and to search for the targets of these known miRNAs, target information of known plant miRNAs was obtained from Plant Non-coding RNA Database (PNRD, access time: December 2016) (http://structuralbiology.cau.edu.cn/PNRD/download.php) using the String Matcher node available in KNIME platform, with settings for cost for deletion, insertion switching and changing selected as 1 for all of them.

psRNATarget online software (http://plantgrn.noble.org/psRNATarget/) was also used to detect the miRNAs from the Puccinellia transcriptome. Default parameters were used for the analysis (2011 version).

2.2.4. 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 down-regulated by boron stress in this work: *O. sativa* boron NIP transporter (UniProtID: Q949A7), *A. thaliana* TIP transporter (UniProtID: O82598), *A. thaliana* BOR6 transporter (UniProtID: Q3E954), *A. thaliana* sugar transporters (UniProtIDs: Q4F7G0, O04249), *O. sativa* sulfate transporter (UniProtID: Q8S317), *O. sativa* anion transporter

(UniProtID: Q652N5), *A. thaliana* inorganic phosphate transporter (UniProtID: Q38954), and *A. thaliana* ABC transporters (UniProtIDs: Q9C9W0, Q8LPJ4, Q7GB25, Q9FWX7, Q9FJH6, Q9M1H3). In addition, 11 boron transporters described in the literature were used in the analysis including those from *O. sativa* (UniProtIDs: Q1ZYR7, Q7X9F3), *A. thaliana* (UniprotID: Q8VYR7, Q9XI23, Q93Z13, Q9M1P7, Q9SUU1, Q9SSG5), *Hordeum vulgare* (UniProtID: M0Z9M9), *Brassica napus* (UniProtID: D5LGA1, D5LG97). Two *A. thaliana* NIP transporters (UniProtID: Q9SV84, Q9SAI4) were also included. Sequences for these proteins were retrieved from the UniProtKB database. *A. thaliana* nuclear transport factor 2 (NTF2) protein (UniprotID: F4J8X6) was included as outgroup. Multiple sequence alignment was conducted with the default settings of ClustalW [109]. All positions with continuous alignment gaps were eliminated using the MEGA 7.0 suite [110]. Phylogenetic tree construction was performed using the maximum likelihood method based on the Le Gascuel substitution model [111]. The Gamma distribution was used to model evolutionary rate differences among sites (with default parameters except for G which was set to 13, 3340). Bootstrap values were inferred from 1000 replicates. In order to compute phylogenetic distances, the boron and other transporters were separated into two groups. Within and between groups distances were calculated by computing the number of amino acid differences per site, averaged over all sequence pairs.

2.2.5. Quantitative Real-Time PCR

The expression of 30 genes randomly selected and ten miRNAs that responded to boron stress in *P. distans* shoots was confirmed by qRT-PCR (quantitative real-time polymerase chain reaction) analysis using three technical replicates from one of the three biological replicates used for RNA-seq analysis. The extracted total RNA was treated with DNase I (Takara, Shiga, Japan). GoTaq® 2-Step RT-qPCR kit (PROMEGA) was used for cDNA synthesis and the resulting cDNAs were amplified in the LightCycler® 480 system (Roche, Basel, Switzerland) using transcript-specific primers (Table 1). The 30 transcripts were selected randomly from the top 50 up and down-regulated transcripts. In addition, primers were used for one known ABC transporter and one known boron transporter [43].

Table 1. Real-Time PCR Primer sequences of *P. distans*

Transcript Name	Forward/Reverse	Sequences
TRINITY_DN174261_c0_g1_i1:0-179	F:	CATATTGATGCCGAACTTAGTG
	R:	GCCTGTAGTCCCAGCTACTC
TRINITY_DN121038_c0_g1_i1:0-299	F:	GGAGTTCTGGGCTGTAGTG
	R:	AGTGCAGTGGCTATTCACA
TRINITY_DN111068_c0_g1_i1:13-283	F:	CTACACGCTGAGGACTCTGT
	R:	GCGTAACTAGAGGGAGCTG
TRINITY_DN220021_c1_g4_i1:0-584	F:	GCACTATACATGTGACGACATT
	R:	CTGTGCTTGAGCTGGTACTAT
TRINITY_DN192927_c1_g1_i1:132-532	F:	ACTTATTGGCAAACTGGTAGAC
	R:	GAGCAATCAAAGATCAGAGG
TRINITY_DN214561_c1_g7_i1:0-308	F:	GATCTATGCTTGAGCTGGTACT
	R:	ATTTTTGCTACAGAGGACACTC
TRINITY_DN215985_c1_g3_i1:4-592	F:	GTGTATCAATACTCCCTTGGAT
	R:	GTTCTGAAATCCTTGTATGTCC
TRINITY_DN224067_c1_g1_i1:0-1399	F:	CTACGTGGTCTTCATTGTGTT
	R:	GAGAAGACTAGCTCCACAGTTC
TRINITY_DN264216_c0_g1_i1:1-257	F:	CTACAAAATGCCAGTATCAGG
	R:	AGAGTACTTCGAGTCTCCCTTC
TRINITY_DN210823_c0_g3_i1:4-269	F:	CCAGATCACCATCCTCAC
	R:	GACGATGACCTCCATCAC
TRINITY_DN216242_c2_g1_i3:1-273	F:	CGTCAGGTATCTTGACAGGTAT
	R:	GGGGAGTTGATAAGTCCATT
TRINITY_DN143038_c0_g1_i1:0-301	F:	TTCTCTCTCCTCACCCTCTT
	R:	GGAAGAGAAGATGGCATAAAG
TRINITY_DN216732_c0_g28_i1:4-191	F:	GGGAGAGATTTTGAAGTTGTG
	R:	CGACATTCAGCAAATAACCT
TRINITY_DN212455_c2_g5_i2:0-590	F:	CAACTACCAGATTACCTTCTGC
	R:	CAAATGTGCACCTCAGTTTA
TRINITY_DN212344_c3_g6_i4:2-174	F:	ACAATGCCGTCTGTGTTAG
	R:	CAGGAGAATAGAAGTGCAAGAT
TRINITY_DN215895_c1_g12_i1:0-174	F:	TGCAAAGACTACAGAAAGAGTG
	R:	CAGGTTGCTCTTGTTTGTCTA
TRINITY_DN221927_c1_g6_i1:0-188	F:	CTCTTTCTTGGGTGTACATTTC
	R:	AAGGCTGTGGGTTTTATTG
TRINITY_DN225496_c0_g7_i1:0-237	F:	CTCTCCCCATATATATACACACC
	R:	TAGTACCGTGCAAAATGTGT
TRINITY_DN220304_c1_g17_i1:0-195	F:	TGTAACTTTCGCAACTTGG
	R:	GGGAACACATTTTTAGCAAC
TRINITY_DN221918_c1_g3_i1:81-282	F:	CAACACACTTACTTAGTAGGGGACA
	R:	GTGCGGCTCCGTGTTATAC
TRINITY_DN222891_c3_g1_i1:0-216	F:	GAACCACTGGGTGTACTGG
	R:	CTTGAAGCAATTTCGATCAC
TRINITY_DN205322_c0_g1_i2:0-204	F:	CGAAGTTCTTCAAGGGGAAGTG
	R:	CGGTCCAACTTATTTGTACGTATCATC
TRINITY_DN216668_c3_g2_i1:0-214	F:	TACTATAGGCGAGCACATGAT
	R:	CTAGCGAAGTTATGCAGTACAA
TRINITY_DN211381_c4_g2_i2:0-214	F:	ATCGTACGTGTTAGGAAGTTCT
	R:	GAAAATCTTTCAGTCCTCAGAC

(Cont. on the next page)

Table 1. (cont.)

TRINITY_DN216505_c2_g1_i1:0-214	F:	ATGTACACGTGGTGTTGCT
	R:	GATTACAGGGTGAATACGTACA
TRINITY_DN215778_c0_g1_i1:24-224	F:	TTACATACGCCACTCACTATACTC
	R:	GTTTTAAGCTGGGCCTGA
TRINITY_DN210741_c3_g13_i1:14-345	F:	CATGATGTTGTCTATCCTGTTC
	R:	GACAAAGCATTTCCTGATGT
TRINITY_DN226817_c0_g1_i1:0-6614	F:	CTACTAGCTGCATTCCTTTCTT
	R:	TGTGTTAGTTAGCACAGGGATA
TRINITY_DN216145_c0_g1_i5:58-633	F:	GATAGGTCCTTGTAGAGCTCAG
	R:	GCACATATAGCTAACCTTCCTC
TRINITY_DN182429_c0_g1_i2:1-380	F:	GTGGATCTGAGAAATGTTTGTG
	R:	TAAAAGAAAGTAGCCAGGAAGC
ABCtransporter	F:	GATCACGAAATTTGGGAGGCGCTA
	R:	ACAGACGGTGCAGTCCTCAAATTC
BoronTransporter	F:	TATAGATTGACAGGCGCTTCCCGT
	R:	CCACGGTTTGTTATGAGCTCGTCC
Actin	F:	CCATTGGTGCTGAGCGTTT
	R:	GCTTCCATCCCAATGAAGGA
miR528	F:	AAGGGGCATGCAGAGGA
	R:	GAAGATTACAGAGGAATGAGGA
miR169e	F:	GATGACTTGCCTATGTCTTCTT
	R:	CAGATCATCCTCTTTGGTAGAT
miRf10218	F:	GAAACCTTGGCTTATATCTTCC
	R:	CTCCGATCCATAAAAAGTGTC
miRf10571	F:	ATCCAGAAGGGCGTGTC
	R:	CAGGCAGGAGAGATTTTACAT
osa-miRf10571-1	F:	GAAGTTTAACCCGTGCTATCTA
	R:	TTATAGTACTCCCTCCGTCCTA
osa-miRf10735	F:	AGTTTCTGCGAGTTTAGGTTAG
	R:	CTTTGTACTAAGTGAGCGACAC
osa-miRf10735-3	F:	GATGTATTCCCTCCGTTTCTA
	R:	GTACTCCCTCCGTTCCTAAATA
osa-miRf10742	F:	CATAACCCTGTTATCAGTGAAG
	R:	ATGATGAACAAGTCCAGACC
miRf11182	F:	GTATGTAAGATGGCAGATACTCC
	R:	GGCTAGATAGATCTTGACATGTAGT
miRf12040	F:	CTCAATGTAGACCTCCAATACA
	R:	ATAGGACGTTTTGGCAAG

CHAPTER 3

RESULTS

3.1. Analysis of the *Puccinellia distans* Transcriptome with RNA-seq

To investigate the transcriptomic response of *P. distans* to boron stress, four cDNA libraries were generated from two replicate mRNA samples from boron-treated and control shoot samples. These libraries were sequenced using Illumina deep-sequencing HiSeq™ 2000. Raw reads of control (90,514,028) and boron-treated stress (89,278,454) samples were generated. Raw reads were cleaned of adapter sequences using the Cutadapt2 tool resulting in 88,663,592 (98.0 %) and 82,494,636 (92.4 %) reads for control and stress samples, respectively.

The cleaned reads from the two samples were treated as input for the Trinity assembler. All reads were mapped to the constructed reference transcriptome individually using the Bowtie2 program. Mapping ratios for control and stress samples were 97.8 % and 97.3 % of cleaned reads, respectively.

Table 2. Summary of transcripts/genes statistics of *P. distans*

Parameter	
Number of Reads (Control + Stress)	179,792,482
Average Raw Read Length (Control + Stress)	125 nt
Average Contig Length	496.73 nt
Median Contig Length	317 nt
N50	609 nt
Total Assembly Size	284,371,845 nt

Based on transcripts that mapped to the reference transcriptome, 284,371,845 bases were assembled from 179,792,482 reads (control + stress) (Table 2). Median and average contig lengths were 317 and ~497 nt, respectively (Table 2). Additionally, the N50 value indicated that at least half the assembled bases were found in contigs that were at least 609 nt long (Table 2).

3.2. Differential Gene Expression between Control and Stress conditions

Differentially expressed transcripts were detected using the RNA-Seq alignments and Cufflinks processing. Cuffdiff tracked the mapped reads and determined the fragments per kb per million mapped reads (FPKM) for each transcript in all samples. These FPKM values were used to detect up and down-regulated transcripts and most differential transcripts were found to be present or absent under control or stress conditions (Figure 1). A total of 2242 of 3312 differentially expressed transcripts were up-regulated (67.7 %) while 1070 of 3312 (32.3 %) were down-regulated (Table 3 and see APPENDIX A).

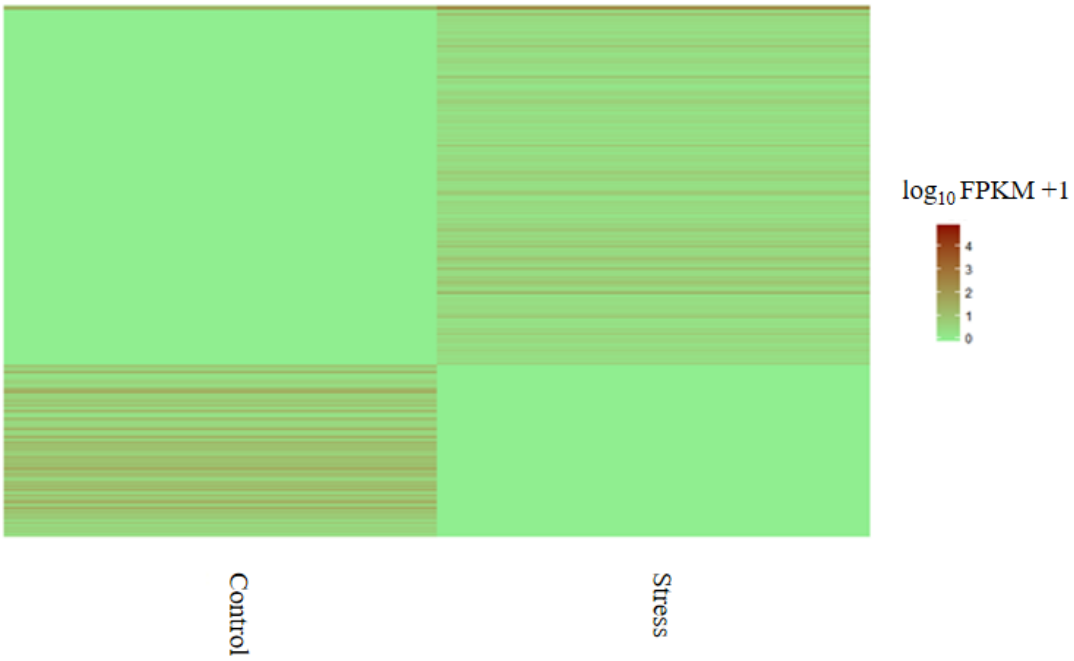

Figure 1. Heat map of log transformed FPKM values of 3312 differentially expressed transcripts from RNA-Seq of control and stress samples. FPKM ranges from 0 (green) to 5 (red).

Table 3. The 100 of FPKM values of differentially expressed transcripts and the FPKM value differences between control and stress groups of *P. distans.*

Transcript Name	Control FPKM	Stress FPKM	Difference between control vs stress
TRINITY_DN174261_c0_g1_i1:0-179	406.0	41806.5	41400.5
TRINITY_DN213899_c1_g5_i1:0-169	365.9	20927.0	20561.1

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Table 3. (cont.)

TRINITY_DN121038_c0_g1_i1:0-299	247.7	18893.6	18645.9
TRINITY_DN174261_c0_g2_i1:0-282	125.0	9078.8	8953.8
TRINITY_DN240298_c0_g1_i1:0-299	96.5	7808.2	7711.7
TRINITY_DN185920_c1_g3_i1:0-163	0.0	3914.9	3914.9
TRINITY_DN111068_c0_g2_i1:14-281	20.0	2281.2	2261.2
TRINITY_DN255535_c0_g1_i1:0-344	34.4	2149.3	2114.8
TRINITY_DN185920_c1_g1_i1:0-270	0.0	1315.7	1315.7
TRINITY_DN237087_c0_g1_i1:45-264	0.0	1102.6	1102.6
TRINITY_DN158617_c0_g3_i1:51-230	0.0	1056.9	1056.9
TRINITY_DN158617_c0_g3_i2:0-344	0.0	1004.4	1004.4
TRINITY_DN169672_c0_g2_i1:37-261	51.8	1028.9	977.1
TRINITY_DN111068_c0_g1_i1:13-283	25.4	988.6	963.2
TRINITY_DN99923_c0_g1_i1:0-171	0.0	845.9	845.9
TRINITY_DN185920_c2_g1_i1:0-1986	0.0	613.7	613.7
TRINITY_DN210950_c1_g3_i1:0-244	35.0	567.0	532.0
TRINITY_DN23532_c0_g1_i1:0-180	0.0	503.9	503.9
TRINITY_DN220021_c1_g4_i1:0-584	104.7	531.0	426.3
TRINITY_DN192927_c1_g1_i1:132-532	82.5	412.1	329.6
TRINITY_DN131860_c0_g2_i1:0-222	0.0	316.0	316.0
TRINITY_DN220021_c1_g2_i1:0-299	28.0	338.5	310.6
TRINITY_DN214561_c1_g7_i1:0-308	32.9	337.8	304.9
TRINITY_DN220021_c1_g4_i2:0-391	33.5	332.8	299.3
TRINITY_DN187809_c1_g2_i1:0-358	19.9	313.4	293.5
TRINITY_DN305698_c0_g1_i1:0-294	0.0	277.0	277.0
TRINITY_DN214561_c1_g18_i1:0-365	27.7	281.3	253.6
TRINITY_DN199272_c0_g3_i1:2-246	0.0	169.0	169.0
TRINITY_DN210741_c3_g13_i1:14-345	19.2	173.4	154.1
TRINITY_DN150574_c0_g1_i1:0-201	0.0	130.1	130.1
TRINITY_DN215985_c1_g3_i1:4-592	23.5	149.8	126.4
TRINITY_DN268357_c0_g1_i1:0-309	0.0	125.5	125.5
TRINITY_DN211139_c12_g1_i2:4-214	0.0	97.7	97.7
TRINITY_DN218921_c2_g11_i1:0-275	0.0	94.5	94.5
TRINITY_DN233649_c0_g1_i1:0-245	0.0	94.4	94.4
TRINITY_DN223632_c5_g9_i1:0-204	0.0	87.0	87.0
TRINITY_DN67858_c0_g1_i1:0-219	0.0	85.6	85.6
TRINITY_DN295912_c0_g1_i1:0-332	0.0	83.3	83.3
TRINITY_DN28983_c0_g1_i1:38-250	0.0	80.8	80.8
TRINITY_DN224067_c1_g1_i1:0-1399	15.8	95.7	79.9
TRINITY_DN206840_c2_g1_i1:32-257	0.0	78.0	78.0
TRINITY_DN194382_c2_g1_i2:0-283	0.0	74.6	74.6
TRINITY_DN156191_c0_g1_i1:0-371	0.0	66.9	66.9
TRINITY_DN211364_c5_g2_i1:0-1033	2.1	68.8	66.7
TRINITY_DN271392_c0_g1_i1:0-479	0.0	66.1	66.1
TRINITY_DN199272_c0_g1_i1:0-245	0.0	65.6	65.6
TRINITY_DN113560_c0_g1_i1:0-238	0.0	63.1	63.1
TRINITY_DN221642_c3_g12_i1:2-232	0.0	57.7	57.7
TRINITY_DN214853_c4_g1_i1:0-762	0.0	57.5	57.5
TRINITY_DN208871_c4_g1_i1:0-352	0.0	55.3	55.3
TRINITY_DN212893_c1_g1_i1:0-261	0.0	53.7	53.7
TRINITY_DN302772_c0_g1_i1:0-218	0.0	52.9	52.9

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Table 3. (cont.)

TRINITY_DN194854_c0_g1_i1:0-255	0.0	52.0	52.0
TRINITY_DN48466_c0_g1_i1:30-384	0.0	51.6	51.6
TRINITY_DN195572_c0_g1_i2:55-342	0.0	49.5	49.5
TRINITY_DN125775_c0_g1_i1:36-294	0.0	48.0	48.0
TRINITY_DN202627_c0_g7_i1:92-353	0.0	45.8	45.8
TRINITY_DN214247_c1_g14_i1:119-359	0.0	44.8	44.8
TRINITY_DN225500_c2_g9_i1:0-236	0.0	40.2	40.2
TRINITY_DN272586_c0_g1_i1:1-261	0.0	39.9	39.9
TRINITY_DN179460_c0_g1_i1:0-227	0.0	39.5	39.5
TRINITY_DN264216_c0_g1_i1:1-257	0.0	38.0	38.0
TRINITY_DN221251_c1_g1_i1:1-258	0.0	34.7	34.7
TRINITY_DN219297_c2_g6_i3:1-241	0.0	34.1	34.1
TRINITY_DN156191_c0_g2_i1:0-342	0.0	32.7	32.7
TRINITY_DN226575_c2_g2_i1:2-256	0.0	32.1	32.1
TRINITY_DN8209_c0_g1_i1:0-419	0.0	31.5	31.5
TRINITY_DN203114_c0_g3_i1:0-256	0.0	30.3	30.3
TRINITY_DN210823_c0_g3_i1:4-269	0.0	30.1	30.1
TRINITY_DN202443_c4_g5_i3:0-460	0.0	29.4	29.4
TRINITY_DN114402_c0_g1_i1:9-274	0.0	29.4	29.4
TRINITY_DN241186_c0_g1_i1:0-1743	2.7	31.9	29.2
TRINITY_DN218938_c0_g2_i1:15-299	0.0	28.3	28.3
TRINITY_DN198354_c0_g2_i1:0-272	0.0	27.2	27.2
TRINITY_DN66009_c0_g1_i1:0-263	0.0	27.0	27.0
TRINITY_DN202443_c4_g4_i2:0-314	0.0	25.9	25.9
TRINITY_DN202443_c4_g4_i3:0-340	0.0	25.5	25.5
TRINITY_DN216242_c2_g1_i3:1-273	0.0	25.3	25.3
TRINITY_DN219995_c1_g16_i1:0-256	0.0	25.1	25.1
TRINITY_DN226626_c0_g8_i1:3-253	0.0	24.8	24.8
TRINITY_DN211426_c2_g4_i3:0-258	0.0	24.3	24.3
TRINITY_DN202443_c4_g1_i1:0-255	0.0	24.2	24.2
TRINITY_DN226234_c5_g4_i1:1-277	0.0	23.9	23.9
TRINITY_DN306660_c0_g1_i1:0-326	0.0	22.6	22.6
TRINITY_DN208926_c0_g1_i1:1-311	0.0	22.3	22.3
TRINITY_DN216505_c0_g1_i2:0-313	0.0	21.9	21.9
TRINITY_DN226592_c4_g15_i1:8-257	0.0	21.5	21.5
TRINITY_DN226529_c6_g13_i2:0-665	3.5	24.2	20.6
TRINITY_DN295721_c0_g1_i1:0-414	0.0	20.4	20.4
TRINITY_DN140573_c0_g2_i1:0-250	0.0	20.3	20.3
TRINITY_DN179003_c1_g2_i1:7-283	0.0	20.1	20.1
TRINITY_DN294520_c0_g1_i1:0-828	0.7	20.8	20.0
TRINITY_DN278982_c0_g1_i1:1-268	0.0	19.7	19.7
TRINITY_DN284184_c0_g1_i1:0-703	0.0	19.5	19.5
TRINITY_DN222292_c3_g2_i1:0-281	0.0	18.7	18.7
TRINITY_DN1712_c0_g1_i1:0-285	0.0	18.6	18.6
TRINITY_DN143038_c0_g1_i1:0-301	0.0	18.6	18.6
TRINITY_DN225050_c0_g11_i1:0-252	0.0	18.3	18.3
TRINITY_DN213110_c2_g2_i4:22-323	0.0	17.0	17.0
TRINITY_DN186267_c2_g1_i1:18-638	0.0	16.9	16.9

3.3. Annotation of Differentially Expressed Transcripts

The number of *P. distans* proteins and annotations available in public data repositories is limited. Therefore, *P. distans* annotation was conducted using the protein databases of two model organisms: *A. thaliana* and *O. sativa*. Both of these have extensive protein sequence resources available in the UniProt knowledgebase repository. In addition, *O. sativa* and *P. distans* belong to the same genus, Gramineae, indicating a close genetic relationship [112]. A total of 3312 transcripts were found to be differentially expressed and, of these, 1652 (49.9 %) mapped to 1107 proteins. Multiple transcripts (a total of 335 transcripts) matching 36 proteins were labelled as conflicting due to matching both up and down-regulated transcripts according to their FPKM values. In order to resolve this, BLAST results were inspected manually. In this way, 36 transcripts with the highest sequence identity and best hit score were kept and the remaining transcripts were discarded as non-annotated. Among the annotated transcripts, 28 matched 21 transporter proteins (Table 4).

Table 4. The annotated 28 transporter transcripts and their annotations

Transcript Name	Difference	Sequence Description
TRINITY_DN87214_c0_g2_i1:31-525	4.90	NTF2_ARATH Nuclear transport factor 2 OS=Arabidopsis thaliana GN=NTF2 PE=1 SV=1
TRINITY_DN175191_c0_g1_i1:30-532	4.38	AB4F_ARATH ABC transporter F family member 4 OS=Arabidopsis thaliana GN=ABCF4 PE=2 SV=1
TRINITY_DN205990_c2_g2_i2:0-577	3.28	BRTL3_ARATH Probable mitochondrial adenine nucleotide transporter BTL3 OS=Arabidopsis thaliana GN=At5g64970 PE=2 SV=1
TRINITY_DN259793_c0_g1_i1:0-487	2.85	F4JUM3_ARATH Sec23 sec24-like transport OS=Arabidopsis thaliana GN=At4g14160 PE=1 SV=1
TRINITY_DN125752_c1_g1_i1:0-599	2.84	SC13B_ARATH transport SEC13 homolog B OS=Arabidopsis thaliana GN=SEC13B PE=1 SV=1

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Table 4. (cont.)

TRINITY_DN172179_c0_g2_i1:0-695	2.55	INT2_ARATH Probable inositol transporter 2 OS=Arabidopsis thaliana GN=INT2 PE=1 SV=1
TRINITY_DN188130_c0_g1_i1:0-1084	2.50	BOR6_ARATH Probable boron transporter 6 OS=Arabidopsis thaliana GN=BOR6 PE=2 SV=2
TRINITY_DN168300_c1_g1_i1:0-634	2.22	PHT21_ARATH Inorganic phosphate transporter 2- chloroplastic OS=Arabidopsis thaliana GN=PHT2-1 PE=1 SV=1
TRINITY_DN157194_c0_g1_i1:0-983	1.88	AB1F_ARATH ABC transporter F family member 1 OS=Arabidopsis thaliana GN=ABCF1 PE=2 SV=1
TRINITY_DN199317_c0_g1_i1:0-1039	1.81	AB1F_ARATH ABC transporter F family member 1 OS=Arabidopsis thaliana GN=ABCF1 PE=2 SV=1
TRINITY_DN70977_c0_g1_i1:0-574	1.77	ECA3_ARATH Calcium-transporting ATPase endoplasmic reticulum-type OS=Arabidopsis thaliana GN=ECA3 PE=2 SV=3
TRINITY_DN177489_c2_g1_i1:0-855	1.75	AB11B_ARATH ABC transporter B family member 11 OS=Arabidopsis thaliana GN=ABCB11 PE=2 SV=1
TRINITY_DN118290_c0_g1_i1:0-808	1.71	Q710Q5_ORYSA H+-transporting ATP synthase (Fragment) OS=Oryza sativa GN=xd1 PE=2 SV=1
TRINITY_DN169703_c0_g1_i1:4-561	1.70	BIG_ARATH Auxin transport BIG OS=Arabidopsis thaliana GN=BIG PE=1 SV=2
TRINITY_DN296026_c0_g1_i1:0-608	1.38	BIG_ARATH Auxin transport BIG OS=Arabidopsis thaliana GN=BIG PE=1 SV=2
TRINITY_DN175191_c2_g2_i1:0-1801	1.17	AB3F_ARATH ABC transporter F family member 3 OS=Arabidopsis thaliana GN=ABCF3 PE=1 SV=1
TRINITY_DN189722_c1_g3_i1:0-1078	1.11	Q710Q5_ORYSA H+-transporting ATP synthase (Fragment) OS=Oryza sativa GN=xd1 PE=2 SV=1
TRINITY_DN124583_c0_g1_i1:0-770	1.02	F4JUM3_ARATH Sec23 sec24-like transport OS=Arabidopsis thaliana GN=At4g14160 PE=1 SV=1
TRINITY_DN214059_c2_g1_i1:33-852	0.99	AB5C_ARATH ABC transporter C family member 5 OS=Arabidopsis thaliana GN=ABCC5 PE=2 SV=2

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Table 4. (cont.)

TRINITY_DN175514_c2_g1_i1:0-886	0.73	SC31B_ARATH transport SEC31 homolog B OS=Arabidopsis thaliana GN=SEC31B PE=1 SV=1
TRINITY_DN168177_c1_g1_i1:0-904	0.68	SC31B_ARATH transport SEC31 homolog B OS=Arabidopsis thaliana GN=SEC31B PE=1 SV=1
TRINITY_DN174719_c1_g1_i1:13-1025	0.61	F4K1Y4_ARATH Nuclear transport factor 2 and RNA recognition motif domain-containing OS=Arabidopsis thaliana GN=At5g60980 PE=1 SV=1
TRINITY_DN192274_c1_g1_i1:0-1907	0.51	AB2E_ARATH ABC transporter E family member 2 OS=Arabidopsis thaliana GN=ABCE2 PE=2 SV=1
TRINITY_DN174719_c1_g1_i3:28-1544	0.48	F4K1Y4_ARATH Nuclear transport factor 2 and RNA recognition motif domain-containing OS=Arabidopsis thaliana GN=At5g60980 PE=1 SV=1
TRINITY_DN174719_c1_g1_i2:0-1650	0.39	F4K1Y4_ARATH Nuclear transport factor 2 and RNA recognition motif domain-containing OS=Arabidopsis thaliana GN=At5g60980 PE=1 SV=1
TRINITY_DN66376_c0_g1_i1:0-338	-5.66	DIT21_ARATH Dicarboxylate transporter chloroplastic OS=Arabidopsis thaliana GN=DIT2-1 PE=1 SV=1
TRINITY_DN200683_c0_g2_i2:72-322	-26.25	Q8S317_ORYSA sulphate transporter OS=Oryza sativa GN=Sultr4-1 PE=2 SV=1
TRINITY_DN223372_c1_g13_i1:16-229	-28.03	ANTR2_ARATH Ascorbate chloroplastic OS=Arabidopsis thaliana GN=PHT4 4 PE=1 SV=1

The annotations for the most differentially expressed transcripts (based on stress and control FPKM values) were listed in Table 5. Transcripts that did not return any match against rice and *A. thaliana* proteins were annotated against the green plant database (Viridiplantae). However, no additional candidate proteins were found at a significant level of match based on alignment length and E-value (data not shown).

Table 5. Transcripts with high differential expression based on FPKM value. Annotated UniProt ID, sequence description and organism [*A. thaliana* (At) and *O. sativa* (Os)] are included.

UniProtID/ Annotation	**Organism**	**Transcript Name**	**Difference ***
F4I600_ARATH/ V-ATPase-related	At	TRINITY_DN213899_c1_g5_i1:0-169	20561.12
H32_ARATH/ Histone	At	TRINITY_DN99923_c0_g1_i1:0-171	845.90
RRAA2_ARATH/ 4-hydroxy-4-methyl-2-oxoglutarate aldolase 2	At	TRINITY_DN220021_c1_g4_i1:0-584	426.28
PSBR_ARATH/Photosystem II 10 kDa chloroplastic	At	TRINITY_DN192927_c1_g1_i1:132-532	329.61
RD22_ARATH/ BURP domain RD22	At	TRINITY_DN131860_c0_g2_i1:0-222	315.95
RRAA3_ARATH/ 4-hydroxy-4-methyl-2-oxoglutarate aldolase 3	At	TRINITY_DN220021_c1_g4_i2:0-391	299.33
RRAA3_ARATH/ 4-hydroxy-4-methyl-2-oxoglutarate aldolase 3	At	TRINITY_DN214561_c1_g18_i1:0-365	253.61
H2B10_ARATH/ Histone	At	TRINITY_DN199272_c0_g3_i1:2-246	168.98
H2B1_ARATH/ Histone	At	TRINITY_DN150574_c0_g1_i1:0-201	130.08
A0A160DR54_ORYSA/ Dirigent (Fragment)	Os	TRINITY_DN215985_c1_g3_i1:4-592	126.38
RL40B_ARATH/ Ubiquitin-60S ribosomal L40-2	At	TRINITY_DN67858_c0_g1_i1:0-219	85.64
H2A1_ARATH/ Probable histone	At	TRINITY_DN28983_c0_g1_i1:38-250	80.83
FRO7_ARATH/ Ferric reduction oxidase chloroplastic	At	TRINITY_DN224067_c1_g1_i1:0-1399	79.87
H32_ARATH/ Histone	At	TRINITY_DN271392_c0_g1_i1:0-479	66.13
H2B3_ARATH/ Histone	At	TRINITY_DN199272_c0_g1_i1:0-245	65.63
H2A1_ARATH/ Probable histone	At	TRINITY_DN113560_c0_g1_i1:0-238	63.07
XTH22_ARATH/ Xyloglucan endotransglucosylase hydrolase 22	At	TRINITY_DN221642_c3_g12_i1:2-232	57.72
H32_ARATH/ Histone	At	TRINITY_DN48466_c0_g1_i1:30-384	51.59
Q01KB9_ORYSA/ Autophagy-related	Os	TRINITY_DN202627_c0_g7_i1:92-353	45.84

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Table 5. (cont.)

ACT3_ARATH/ Actin-3	At	TRINITY_DN272586_c0_g1_i1:1-261	39.90
SYHM_ARATH/ Histidine-tRNA chloroplastic mitochondrial	At	TRINITY_DN179460_c0_g1_i1:0-227	39.49
COX3_ARATH/ Cytochrome c oxidase subunit 3	At	TRINITY_DN264216_c0_g1_i1:1-257	38.02
H4_ARATH/ Histone H4	At	TRINITY_DN8209_c0_g1_i1:0-419	31.53
Q949A7_ORYSA/ aquaporin (Fragment)	Os	TRINITY_DN210823_c0_g3_i1:4-269	30.12
H2A1_ARATH/ Probable histone	At	TRINITY_DN202443_c4_g5_i3:0-460	29.44
FRS5_ARATH/ FAR1-RELATED SEQUENCE 5	At	TRINITY_DN114402_c0_g1_i1:9-274	29.40
H2A1_ARATH/ Probable histone	At	TRINITY_DN202443_c4_g4_i2:0-314	25.88
H2A1_ARATH/ Probable histone	At	TRINITY_DN202443_c4_g4_i3:0-340	25.53
Q9LK47_ARATH/ AT3g23700 MYM9_3	At	TRINITY_DN219995_c1_g16_i1:0-256	25.08
A0A0K0WRF9_ORYSA/ NBS-LRR type R	Os	TRINITY_DN226626_c0_g8_i1:3-253	24.76
H2A1_ARATH/ Probable histone	At	TRINITY_DN202443_c4_g1_i1:0-255	24.25
NEN3_ARATH/ NEN3	At	TRINITY_DN226592_c4_g15_i1:8-257	21.50
RL341_ARATH/ 60S ribosomal L34-1	At	TRINITY_DN295721_c0_g1_i1:0-414	20.41
F4HS76_ARATH/ Glycerol kinase	At	TRINITY_DN140573_c0_g2_i1:0-250	20.29
RL142_ARATH/ 60S ribosomal L14-2	At	TRINITY_DN179003_c1_g2_i1:7-283	20.12
RL74_ARATH/ 60S ribosomal L7-4	At	TRINITY_DN294520_c0_g1_i1:0-828	20.02
F4IJE1_ARATH/ Phox domain-containing	At	TRINITY_DN278982_c0_g1_i1:1-268	19.68
COX2_ARATH/ Cytochrome c oxidase subunit 2	At	TRINITY_DN284184_c0_g1_i1:0-703	19.48
Y1457_ARATH/ Acyltransferase chloroplastic	At	TRINITY_DN222292_c3_g2_i1:0-281	18.69

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Table 5. (cont.)

Q9LTW8_ARATH/ CTP synthase	At	TRINITY_DN1712_c0_g1_i1:0-285	18.65
A0A0U2JFK9_ORYSA/ NBS-LRR-like resistance	Os	TRINITY_DN213110_c2_g2_i4:22-323	16.98
UBQ3_ARATH/ Polyubiquitin 3	At	TRINITY_DN186267_c2_g1_i1:18-638	16.88
ACT11_ARATH/ Actin-11	At	TRINITY_DN204305_c3_g6_i2:0-375	16.85
A8MRY7_ARATH/ WD-40 PCN	At	TRINITY_DN224769_c1_g7_i1:4-287	15.55
Q9STF8_ARATH/ PR-6ase inhibitor family	At	TRINITY_DN127914_c0_g2_i1:0-296	15.37

*Difference: FPKM (Fragments per Kilobase of Exon per Million Fragments Mapped) difference between control and stress conditions.

Functional annotation was performed using Blast2GO and QuickGO based on gene ontology (GO). Cellular component, molecular function, and biological process were analyzed as GO terms for the 1107 annotated proteins. According to the ontology analysis, 906 proteins (81.8 %) were annotated to cellular components. 885 (80.0 %) and 848 proteins (76.6 %) were annotated with their molecular functions and biological processes, respectively.

3.4. Cellular Components

Cellular component analysis connected 906 proteins (81.8%) to at least one cellular component-associated term (Table 6). Because excess boron was trafficked from the plasma membrane to other organelles such as the vacuole and cell wall to avoid boron toxicity, only these cellular component-associated GO terms are discussed here. A total of 14.8% of proteins were associated with the integral component of the membrane, plasma membrane, vacuolar membrane, mitochondrial inner membrane and endoplasmic reticulum membrane. In addition, 1.4% of proteins were related to the cell wall, 0.8% were localized in the apoplast and 1.5% were in the vacuole.

Table 6. Cellular component categories which are most related to boron hyper-accumulation in *P. distans*

Cellular component GOTerm	Percentage of proteins
Cytosol	7.9%
Nucleus	7.8%
Integral component of membrane	5.9%
Plasma membrane	5.0%
Chloroplast	4.7%
Nucleolus	3.9%
Plasmodesmata	3.3%
Mitochondrion	3.1%
Vacuolar membrane	2.0%
Cytosolic ribosome	1.8%
Endoplasmic reticulum	1.7%
Vacuole	1.5%
Golgi apparatus	1.5%
Cell wall	1.4%
Chloroplast envelope	1.4%
Chloroplast stroma	1.3%
Ribosome	1.1%
Mitochondrial inner membrane	1.0%
Endoplasmic reticulum membrane	0.9%
Apoplast	0.8%
Plastid	0.6%

3.5. Biological Processes

Biological process annotations fell into seven main categories: response to stimulus, cellular process, metabolic process, localization, reproductive process, developmental process and signaling (Table 7). Proteins related to plant responses to hormones and stress conditions such as salt, various ions, cold, heat, oxidative stress, and water deprivation were identified. Our findings indicated that not only translation and transcription but also protein folding and turnover, cell wall organization and biogenesis, and ion dependent redox homeostasis were regulated under excess boron. Both anabolic processes such as the malate-fumarate pathway and catabolic processes such as protein and lignin pathways were altered under stress conditions. Proteins that play roles in signal transduction, in particular plant hormone-mediated and sugar-mediated signaling pathways were also differentially regulated.

Table 7. Biological process main and sub categories which are most related to boron hyper-accumulation in *P. distans*

Biological Process GOTerm	**Percentage of Proteins**
Response to stimulus	
Response to salt stress	1.30%
Response to cadmium ion	1.30%
Response to cold	1.20%
Response to abscisic acid	0.90%
Response to heat	0.70%
Defense response to bacterium	0.70%
Response to cytokinin	0.70%
Response to oxidative stress	0.70%
Response to water deprivation	0.60%
Defense response	0.30%
Cellular response to DNA damage stimulus	0.30%
Response to light stimulus	0.30%
Response to high light intensity	0.30%
Response to glucose	0.20%
Response to zinc ion	0.20%
Cellular response to abscisic acid stimulus	0.20%
Response to stress	0.20%
Defense response to fungus, incompatible interaction	0.20%
Response to UV-B	0.20%
Response to auxin	0.20%
Cellular response to nitrogen starvation	0.10%
Response to L-ascorbic acid	0.10%
Response to ethylene	0.10%
Cellular response to oxidative stress	0.10%
Plant-type hypersensitive response	0.10%
Cellular response to potassium ion starvation	0.10%
Hyperosmotic salinity response	0.10%
Cellular response to phosphate starvation	0.10%
Response to jasmonic acid	0.10%
Positive regulation of vernalization response	0.10%
Hyperosmotic response	0.10%
Cellular response to calcium ion	0.10%
Response to extracellular stimulus	0.10%
Response to gibberellin	0.10%
Cellular response to stress	0.10%
Response to salicylic acid	0.10%
Response to cobalt ion	0.10%
Response to hypoxia	0.10%
Drought recovery	0.10%
Response to reactive oxygen species	0.10%
Detection of hypoxia	0.10%
Cellular Process	
Translation	2.90%

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Table 7. (cont.)

Regulation of transcription, DNA-templated	2.00%
Transcription, DNA-templated	1.70%
Protein folding	0.60%
Protein ubiquitination	0.60%
Tricarboxylic acid cycle	0.40%
Cell redox homeostasis	0.40%
Cell wall organization	0.30%
Trichome morphogenesis	0.20%
Chaperone-mediated protein folding	0.20%
Regulation of stomatal closure	0.20%
Cellular zinc ion homeostasis	0.10%
Vacuole fusion	0.10%
Cellular potassium ion homeostasis	0.10%
Cell wall biogenesis	0.10%
Chaperone mediated protein folding requiring cofactor	0.10%
Metabolic Process	
Protein catabolic process	0.30%
Oxidation-reduction process	0.20%
Malate metabolic process	0.20%
Secondary metabolite biosynthetic process	0.20%
Fumarate metabolic process	0.20%
Hydrogen peroxide catabolic process	0.20%
Glutathione metabolic process	0.20%
Reactive oxygen species metabolic process	0.20%
Iron-sulfur cluster assembly	0.10%
Cullin deneddylation	0.10%
Sucrose biosynthetic process	0.10%
Oxylipin biosynthetic process	0.10%
Cell wall mannoprotein biosynthetic process	0.10%
Lignin biosynthetic process	0.10%
Lignin catabolic process	0.10%
Oxaloacetate metabolic process	0.10%
Glyoxylate metabolic process	0.10%
2-oxoglutarate metabolic process	0.10%
Aspartate metabolic process	0.10%
Localization	
Protein transport	1.10%
Vesicle-mediated transport	0.60%
ER to Golgi vesicle-mediated transport	0.30%
Transport	0.20%
Auxin polar transport	0.20%
Anion transmembrane transport	0.20%
Ion transport	0.20%
Potassium ion transport	0.10%
Protein targeting to vacuole	0.10%
Potassium ion import	0.10%

(Cont. on the next page)

Table 7. (cont.)

Sulfate transport	0.10%
Zinc II ion transmembrane transport	0.10%
Oxaloacetate transport	0.10%
Phosphate ion transmembrane transport	0.10%
Malate transmembrane transport	0.10%
Sodium ion transport	0.10%
Myo-inositol transport	0.10%
Reproductive Process	
Embryo development ending in seed dormancy	0.90%
Pollen tube growth	0.20%
Developmental Process	
Root development	0.50%
Seed development	0.20%
Signaling	
Signal transduction	0.30%
Abscisic acid-activated signaling pathway	0.30%
Negative regulation of abscisic acid-activated signaling pathway	0.30%
Sugar mediated signaling pathway	0.20%
Gibberellin mediated signaling pathway	0.10%

3.6. Molecular Functions

Molecular function annotation of transcripts resulted in three main categories: transporter activity, catalytic activity and binding (Table 8). The most abundant molecular function categories were binding activity (39%) and catalytic activity (67%). Binding activity included copper, zinc, calcium, manganese, cobalt and ferritin binding indicating that excess boron alters signal transduction and vesicle trafficking in the cell. Catalytic activities of enzymes such as serine/threonine kinase, GTPase, ATPase, H^+ transporting ATP synthase, and oxidoreductase were regulated under boron stress. Differentially expressed transporters included proton, glucose, malate, inorganic phosphate, L-ascorbic acid, L-glutamate, auxin, and ion transporters. Transporters for minerals including calcium, sulfate, and manganese were also identified in this category. Moreover, three previously identified boron transporters were found: *A. thaliana* BOR6 putative boron transporter protein (Transcript Name: TRINITY_DN188130_c0_g1_i1:0-1084), *A. thaliana* TIP1-3 protein (Transcript Name: TRINITY_DN222891_c3_g1_i1:0-216), and *O. sativa* NIP protein (Transcript Name: TRINITY_DN210823_c0_g3_i1:4-269).

Table 8. Molecular function main and subcategories which were most related to boron hyperaccumulation in *P. distans*.

Molecular Function GOTerm	Percentage of proteins
Binding	39.00 %
ATP binding	9.90 %
Metal ion binding	4.60 %
RNA binding	3.80 %
DNA binding	3.70 %
Zinc ion binding	3.20 %
GTP binding	1.60 %
Translation initiation factor activity	1.60 %
Nucleic acid binding	1.50 %
Copper ion binding	1.40 %
Transcription factor activity, sequence-specific DNA binding	1.10 %
ADP binding	1.00 %
Heme binding	1.00 %
Calcium ion binding	0.60 %
Iron ion binding	0.50 %
Magnesium ion binding	0.30 %
HSP90 protein binding	0.20 %
4 iron, 4 sulfur cluster binding	0.20 %
Cobalt ion binding	0.10 %
Manganese ion binding	0.10 %
2 iron, 2 sulfur cluster binding	0.10 %
Four-way junction DNA binding	0.10 %
Calmodulin binding	0.10 %
Quinone binding	0.10 %
Iron-sulfur cluster binding	0.10 %
Catalytic activity	67.00 %
Structural constituent of ribosome	4.90 %
Protein serine/threonine kinase activity	1.30 %
Protein kinase activity	1.00 %
GTPase activity	0.90 %
ATP-dependent RNA helicase activity	0.80 %
ATPase activity	0.80 %
NADH dehydrogenase (ubiquinone) activity	0.60 %
Proton-transporting ATP synthase activity, rotational mechanism	0.60 %
Structural molecule activity	0.60 %
Oxidoreductase activity	0.60 %
Kinase activity	0.50 %
Signal transducer activity	0.30 %
Peroxidase activity	0.30 %
Metalloendopeptidase activity	0.30 %
Cytochrome-c oxidase activity	0.30 %
Fumarate hydratase activity	0.20 %
Metalloaminopeptidase activity	0.10 %
Calcium-dependent cysteine-type endopeptidase activity	0.10 %

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Table 8. (cont.)

Glutathione transferase activity	0.10 %
Peroxiredoxin activity	0.10 %
Oxidoreductase activity, acting on the CH-CH group of donors, with a flavin as acceptor	0.10 %
Glutathione peroxidase activity	0.10 %
Calcium-transporting ATPase activity	0.10 %
Electron transporter, transferring electrons from COQH2-cytochrome c reductase complex and cytochrome c oxidase complex activity	0.10 %
L-lactate dehydrogenase activity	0.10 %
L-malate dehydrogenase activity	0.10 %
Metallopeptidase activity	0.10 %
Ferric-chelate reductase activity	0.10 %
Substrate-specific transmembrane transporter activity	0.10 %
Oxaloacetate decarboxylase activity	0.10 %
Manganese-transporting ATPase activity	0.10 %
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.10 %
Malate dehydrogenase (decarboxylating) (NADP+) activity	0.10 %
Oxidoreductase activity, acting on the CH-CH group of donors	0.10 %
Oxalate decarboxylase activity	0.10 %
Oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	0.10 %
L-ascorbate peroxidase activity	0.10 %
Cytochrome-c peroxidase activity	0.10 %
Glutamate decarboxylase activity	0.10 %
Myo-inositol	0.10 %
Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	0.10 %
Nuclear export signal receptor activity	0.10 %
Oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor	0.10 %
Aspartate-tRNA ligase activity	0.10 %
Malate dehydrogenase (decarboxylating) (NAD+) activity	0.10 %
Oxidoreductase activity, oxidizing metal ions	0.10 %
Protein tyrosine/serine/threonine phosphatase activity	0.10 %
Transporter activity	9.00 %
Transporter activity	0.40 %
Glucose transmembrane transporter activity	0.20 %
Sugar:proton symporter activity	0.20 %
Inorganic phosphate transmembrane transporter activity	0.20 %
Malate transmembrane transporter activity	0.10 %
L-ascorbic acid transporter activity	0.10 %
Secondary active sulfate transmembrane transporter activity	0.10 %
Antiporter activity	0.10 %
Voltage-gated ion channel activity	0.10 %
Anion:anion antiporter activity	0.10 %
Auxin efflux transmembrane transporter activity	0.10 %
Outward rectifier potassium channel activity	0.10 %

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Table 8. (cont.)

Ion transmembrane transporter activity	0.10 %
Ion channel activity	0.10 %
Channel regulator activity	0.10 %
Inorganic anion exchanger activity	0.10 %
Anion transmembrane transporter activity	0.10 %
Inward rectifier potassium channel activity	0.10 %
L-glutamate transmembrane transporter activity	0.10 %
Zinc ion transmembrane transporter activity	0.10 %
Porin activity	0.10 %

Annotated transcripts were uploaded to the KEGG system for metabolic pathway analysis. A total of 756 proteins were found to have interactions with pathways in the KEGG database (Table 9). In this database, 327 (43.2%) proteins fell into the general metabolism category. Of these metabolism-related proteins, 66 were involved in carbon metabolism, 64 in energy metabolism, 50 in amino acid metabolism, 31 in nucleic acid metabolism, 24 in lipid metabolism and 7 in secondary metabolism. The remaining 34 proteins were placed in other metabolism-related subcategories. A total of 387 proteins (51.2%) were in the genetic information process category. Of these, the majority (184) fell into the translation subcategory. The protein folding category was the second most populous group in genetic information processes with 98 proteins. Environmental information processes were also observed for 152 proteins (20.1%) with 148 proteins matching signal transduction and four matching membrane transport. Finally, 152 proteins (20.1%) were associated with cellular processes. In this category, two subgroups resulted in high protein matches: 86 proteins in the cellular growth and death subcategory and 42 proteins in the transport and catabolism subcategory.

Table 9. Distribution of KEGG functional annotation of the *P. distans* transcriptome

PathwayID Pathways	Amount of unigene	Percentage
03010 Ribosome	96	6.0
03040 Spliceosome	43	2.7
00190 Oxidative phosphorylation	37	2.3
04141 Protein processing in endoplasmic reticulum	34	2.1
03013 RNA transport	32	2.0
01200 Carbon metabolism	29	1.8
04110 Cell cycle	25	1.6
03008 Ribosome biogenesis in eukaryotes	23	1.4
03050 Proteasome	22	1.4

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Table 9. (cont.)

03015 mRNA surveillance pathway	18	1.1
00230 Purine metabolism	17	1.1
03018 RNA degradation	17	1.1
03030 DNA replication	17	1.1
04120 Ubiquitin mediated proteolysis	16	1.0
00970 Aminoacyl-tRNA biosynthesis	15	0.9
01230 Biosynthesis of amino acids	14	0.9
00020 Citrate cycle	14	0.9
00240 Pyrimidine metabolism	14	0.9
04144 Endocytosis	14	0.9
03420 Nucleotide excision repair	13	0.8
04151 PI3K-Akt signaling pathway	13	0.8
04113 Meiosis - yeast	13	0.8
05200 Pathways in cancer	13	0.8
04910 Insulin signaling pathway	12	0.7
05168 Herpes simplex infection	12	0.7
00270 Cysteine and methionine metabolism	11	0.7
00010 Glycolysis / Gluconeogenesis	10	0.6
05166 HTLV-I infection	10	0.6
03430 Mismatch repair	9	0.6
04152 AMPK signaling pathway	9	0.6
04626 Plant-pathogen interaction	9	0.6
05034 Alcoholism	9	0.6
00710 Carbon fixation in photosynthetic organisms	8	0.5
03060 Protein export	8	0.5
04011 MAPK signaling pathway - yeast	8	0.5
04142 Lysosome	8	0.5
04810 Regulation of actin cytoskeleton	8	0.5
04922 Glucagon signaling pathway	8	0.5
04915 Estrogen signaling pathway	8	0.5
04260 Cardiac muscle contraction	8	0.5
04212 Longevity regulating pathway - worm	8	0.5
00030 Pentose phosphate pathway	7	0.4
00051 Fructose and mannose metabolism	7	0.4
00680 Methane metabolism	7	0.4
04013 MAPK signaling pathway - fly	7	0.4
04016 MAPK signaling pathway - plant	7	0.4
04066 HIF-1 signaling pathway	7	0.4
04022 cGMP - PKG signaling pathway	7	0.4
04150 mTOR signaling pathway	7	0.4
04075 Plant hormone signal transduction	7	0.4
04145 Phagosome	7	0.4
04139 Mitophagy - yeast	7	0.4
04612 Antigen processing and presentation	7	0.4
04914 Progesterone-mediated oocyte maturation	7	0.4
04728 Dopaminergic synapse	7	0.4

(Cont. on the next page)

Table 9. (cont.)

05110 Vibrio cholerae infection	7	0.4
05134 Legionellosis	7	0.4
00620 Pyruvate metabolism	6	0.4
00720 Carbon fixation pathways in prokaryotes	6	0.4
03020 RNA polymerase	6	0.4
03410 Base excision repair	6	0.4
04310 Wnt signaling pathway	6	0.4
04068 FoxO signaling pathway	6	0.4
04020 Calcium signaling pathway	6	0.4
04214 Apoptosis - fly	6	0.4
04530 Tight junction	6	0.4
04621 NOD-like receptor signaling pathway	6	0.4
04666 Fc gamma R-mediated phagocytosis	6	0.4
04721 Synaptic vesicle cycle	6	0.4
05322 Systemic lupus erythematosus	6	0.4
04931 Insulin resistance	6	0.4
05162 Measles	6	0.4
05164 Influenza A	6	0.4
05161 Hepatitis B	6	0.4
00520 Amino sugar and nucleotide sugar metabolism	5	0.3
00630 Glyoxylate and dicarboxylate metabolism	5	0.3
00350 Tyrosine metabolism	5	0.3
00480 Glutathione metabolism	5	0.3
02020 Two-component system	5	0.3
04014 Ras signaling pathway	5	0.3
04390 Hippo signaling pathway	5	0.3
04657 IL-17 signaling pathway	5	0.3
04919 Thyroid hormone signaling pathway	5	0.3
04727 GABAergic synapse	5	0.3
04213 Longevity regulating pathway - multiple species	5	0.3
05205 Proteoglycans in cancer	5	0.3
05210 Colorectal cancer	5	0.3
05215 Prostate cancer	5	0.3
05120 Epithelial cell signaling in Helicobacter pylori infection	5	0.3
05152 Tuberculosis	5	0.3
00100 Steroid biosynthesis	4	0.2
03440 Homologous recombination	4	0.2
03460 Fanconi anemia pathway	4	0.2
04015 Rap1 signaling pathway	4	0.2
04010 MAPK signaling pathway	4	0.2
04012 ErbB signaling pathway	4	0.2
04350 TGF-beta signaling pathway	4	0.2
04072 Phospholipase D signaling pathway	4	0.2
04024 cAMP signaling pathway	4	0.2
04146 Peroxisome	4	0.2

(Cont. on the next page)

Table 9. (cont.)

04210 Apoptosis	4	0.2
04115 p53 signaling pathway	4	0.2
04062 Chemokine signaling pathway	4	0.2
03320 PPAR signaling pathway	4	0.2
04918 Thyroid hormone synthesis	4	0.2
04261 Adrenergic signaling in cardiomyocytes	4	0.2
04961 Endocrine and other factor-regulated calcium reabsorption	4	0.2
04722 Neurotrophin signaling pathway	4	0.2
04360 Axon guidance	4	0.2
04211 Longevity regulating pathway - mammal	4	0.2
05231 Choline metabolism in cancer	4	0.2
05202 Transcriptional misregulation in cancers	4	0.2
05206 MicroRNAs in cancer	4	0.2
05323 Rheumatoid arthritis	4	0.2
05131 Shigellosis	4	0.2
05160 Hepatitis C	4	0.2
01524 Platinum drug resistance	4	0.2
01210 2-Oxocarboxylic acid metabolism	3	0.2
01212 Fatty acid metabolism	3	0.2
00052 Galactose metabolism	3	0.2
00500 Starch and sucrose metabolism	3	0.2
00196 Photosynthesis - antenna proteins	3	0.2
00071 Fatty acid degradation	3	0.2
00561 Glycerolipid metabolism	3	0.2
00564 Glycerophospholipid metabolism	3	0.2
00590 Arachidonic acid metabolism	3	0.2
00250 Alanine, aspartate and glutamate metabolism	3	0.2
00280 Valine, leucine and isoleucine degradation	3	0.2
00330 Arginine and proline metabolism	3	0.2
00430 Taurine and hypotaurine metabolism	3	0.2
00450 Selenocompound metabolism	3	0.2
00510 N-Glycan biosynthesis	3	0.2
00830 Retinol metabolism	3	0.2
00860 Porphyrin and chlorophyll metabolism	3	0.2
00130 Ubiquinone and other terpenoid-quinone biosynthesis	3	0.2
04341 Hedgehog signaling pathway - Fly	3	0.2
04391 Hippo signaling pathway -fly	3	0.2
04070 Phosphatidylinositol signaling system	3	0.2
04510 Focal adhesion	3	0.2
04623 Cytosolic DNA-sensing pathway	3	0.2
04659 Th17 cell differentiation	3	0.2
04920 Adipocytokine signaling pathway	3	0.2
04921 Oxytocin signaling pathway	3	0.2
04916 Melanogenesis	3	0.2
04270 Vascular smooth muscle contraction	3	0.2

(Cont. on the next page)

Table 9. (cont.)

04966 Collecting duct acid secretion	3	0.2
04720 Long-term potentiation	3	0.2
05230 Central carbon metabolism in cancer	3	0.2
05211 Renal cell carcinoma	3	0.2
05224 Breast cancer	3	0.2
05014 Amyotrophic lateral sclerosis	3	0.2
05416 Viral myocarditis	3	0.2
05100 Bacterial invasion of epithelial cells	3	0.2
01521 EGFR tyrosine kinase inhibitor resistance	3	0.2

3.7. Evolutionary Relationships among Transporters

O. sativa and *A. thaliana* are not boron hyperaccumulating plants; therefore, boron transporters in *P. distans* may have been annotated to other ion transporters in these species. Phylogenetic analysis was used to determine the degree of evolutionary similarity between known boron transporters (from *O. sativa, A. thaliana, B. napus,* and *H. vulgare*) and the annotated *A. thaliana* and *O. sativa* boron (BOR6), aquaporin (NIP and TIP), sugar, sulfate, anion, inorganic phosphate, and ABC transporters from our dataset (Figure 2). A total of 28 amino acid sequences were compared over 160 positions. The samples included an *A. thaliana* nuclear transport factor protein as outgroup. The phylogenetic analysis indicated that the identified sugar, sulfate, anion, inorganic phosphate and ABC transporter proteins were distinct from boron transporters which were also distinct from aquaporins. The mean p-distance was 30% between boron transporters and 60% between aquaporins. Wakuta et al. (2015) examined the evolutionary relationships among boron transporters in terrestrial plants [62]. According to this work, boron transporters fell into two major and one minor clade that may reflect their physiological differences. Clade I contained transporters such as BOR1 that are important under limited boron conditions and are responsible for directional export of boron in the plant. Clade II contained transporters such as BOR6 that are important under high boron conditions and are responsible for boron exclusion. Our phylogenetic analysis agreed with that of Wakuta et al. (2015) in separating the BOR1, 2 and 3 homologs from BOR6 and 7.

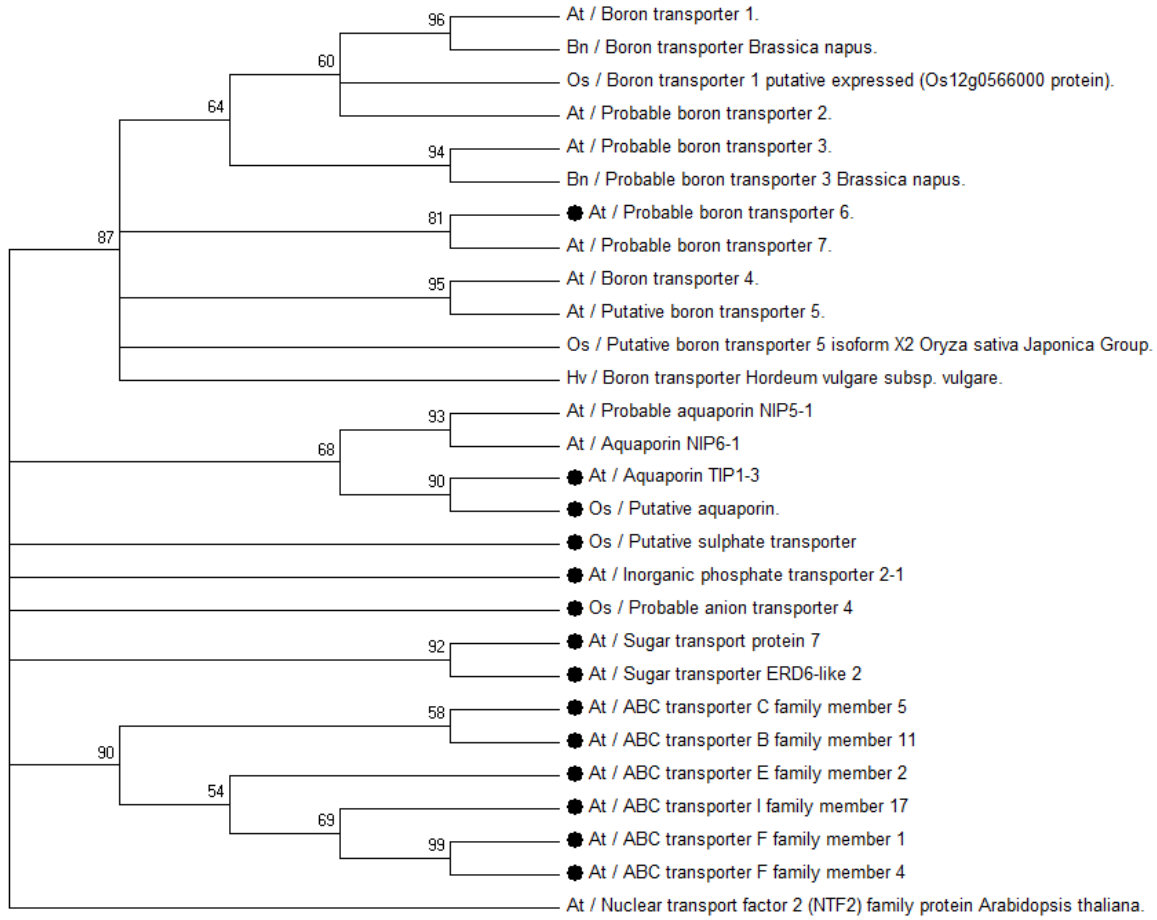

Figure 2. Maximum Likelihood phylogenetic tree representing the evolutionary relationship of boron TIP and NIP transporters and other ion and molecule transporters of several species. *A. thaliana* NTF2 was used as outgroup. The evolutionary history was inferred using the maximum likelihood method based on the Le Gascuel 2008 model. The tree with the highest log likelihood (-8734, 1061) is shown. Bootstrap values are indicated at the branches. There were a total of 160 positions in the final dataset. ● indicates the annotated transcripts from this study.

3.8. qRT-PCR Results of Transcript Analysis

A total of 32 transcripts were selected for verification of their expression levels using quantitative real time-PCR (qRT-PCR). Transcripts were chosen randomly based on their differential expression between control and stress samples. The qRT-PCR analysis was performed according to the 2-ΔΔCt method. Up and down-regulated transcripts are shown in Figure 3. The most significant five up-regulated transcripts were annotated to the following proteins: an aquaporin of *O. sativa* (DN210823); a photosystem II protein in *Arabidopsis* (DN192927); a 4-hydroxy-4-methyl-2-

oxoglutarate aldolase in *A. thaliana* (DN220021); a ferric reduction oxidase in *A. thaliana* (DN224067); and FRIGIDA, which is required for FLC (Flowering Locus C) activity in *A. thaliana* (DN111068)(Figure 3a). Significant down-regulation was observed for four transcripts: three of which were annotated to have oxidoreductase activities in *O. sativa* (DN212455, DN205322, DN216505) and one that was annotated to aquaporin TIP1-3 in *A. thaliana* (DN222891).

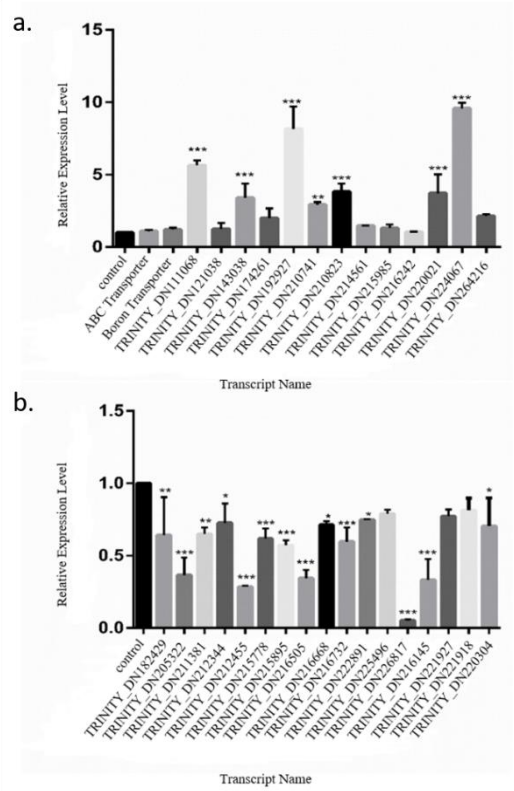

Figure 3. Real-time PCR results of *P. distans* transcriptome for 32 selected transcripts. (a) Fifteen of the transcripts were confirmed to be up-regulated; while 17 were confirmed to be down-regulated (b) Actin was used as internal control and error bars indicate standard deviation of three technical repeats. *** indicates significant difference at $p \leq 0.001$, ** indicates significant difference at $p \leq 0.01$ and * indicates significant difference at $p \leq 0.05$ with respect to control.

3.9. Identification of miRNAs

Hairpin predictions were performed by searching which model was the more accurate (Figure 4). Distribution of model performances was also generated to predict mature miRNAs (Figure 5). A total of 74 predictions from 24,657 sequences were found to be affected by boron treatment and to show similarity to already known sequences with maximum distance score of 4 (Table 10). For instance, maximum distance 2 was

identified for osa-miRf11603-npr and osa-miRf12040-npr. The targets of these miRNA were calcium/calmodulin dependent protein kinases for osa-miRf11603 and mRNA cap guanine-N7 methyltransferase 2 for osa-miRf12040-npr.

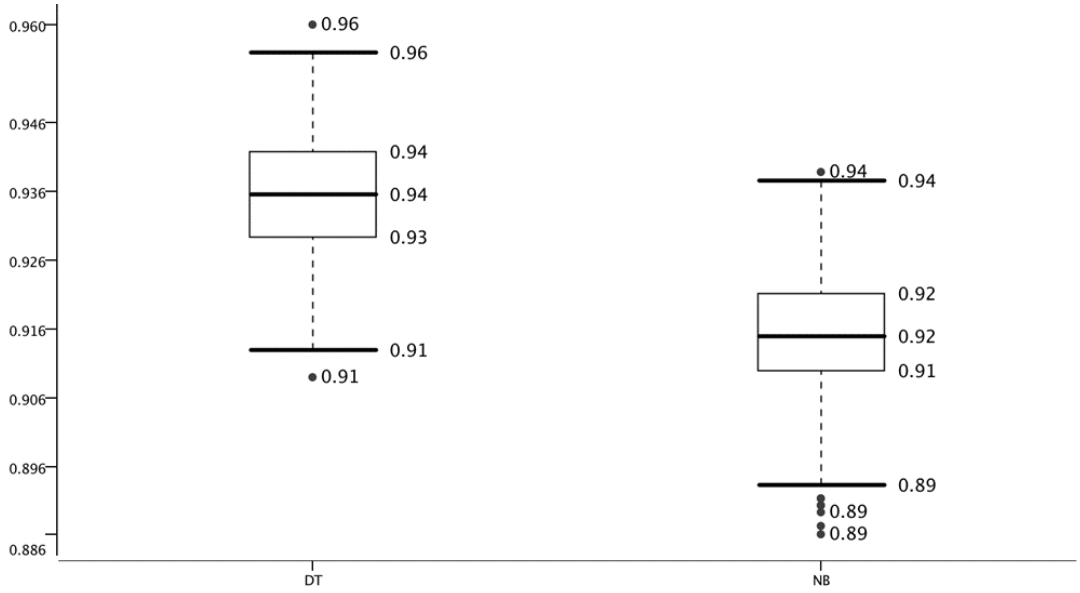

Figure 4. Accuracy distribution of models generated for hairpin prediction (DT: Decision Tree, NB: Naive Bayes).

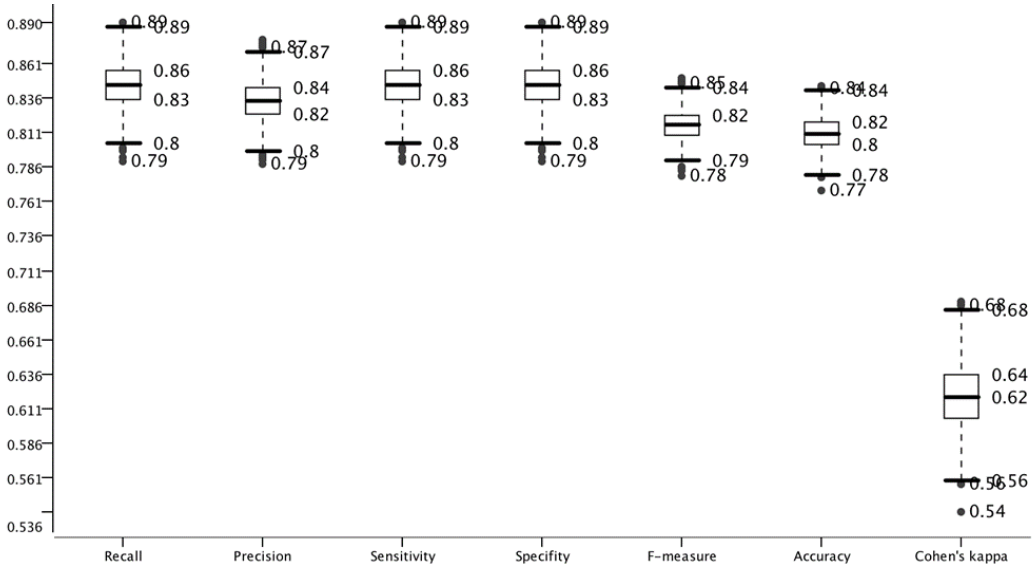

Figure 5. Distribution of model performances generated for mature prediction (Random Forest Learner)

Table 10. Mature miRNAs from *P. distans* transcriptome

ncRNA	OurmiRNAs	knownmiRNAsequence	Distance
osa-miRf12040-npr	UACUCCCUCCGUUCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	2
osa-miRf12040-npr	UACUCCCUCUGUCCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	2
osa-miRf12040-npr	UACUCUCUCCGUCCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	2
osa-miRf11603-npr	UACUCCCUCCGUCCCAAAAUAAAU	CUACUCCCUCCGUCCCAAAAUAAGU	2
osa-miRf12040-npr	UACUCCCUCUGUCCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	2
osa-miRf11612-npr	UACUCCCUCUGUCCCAAAAUAUAA	UACUCCCUCCGUCCCUAAAUAUA	3
osa-miRf10218-npr	AUUACUCCCUCCGAUCCUAAAAAA	UAUACUCCCUCCGUCCCAAAAAA	3
osa-miRf10497-npr	CUCCGUCCCAUAAUAUAAGAUAUU	CUCCGUCCCUAAAUAUAAGGGAUU	3
osa-miRf12039-npr	CGUACUCCCUCCGUUCCAUAAUAU	AGGUACUCCCUCCGUCCCAUAAUAU	3
bdi-miR169k	UAGCCAAGGAUGACUUGCCUAUGU	UAGCCAAGGAUGAUUUGCCUGU	3
sit-miR169e	UAGCCAAGGAUGACUUGCCUAUGU	UAGCCAAGGAUGAUUUGCCUGU	3
bdi-miR169k	UAGCCAAGGAUGACUUGCCUAUGU	UAGCCAAGGAUGAUUUGCCUGU	3
sit-miR169e	UAGCCAAGGAUGACUUGCCUAUGU	UAGCCAAGGAUGAUUUGCCUGU	3
osa-miRf11954-npr	GGUUUUAGUACUCCCUCCGUCCCA	UUUAGUACUCCCUCCGUCCCA	3
osa-miRf10560-npr	ACCUUUUGUCCCGGUUCGUGUUAU	ACUUUAGUCCCGGUUGGUGUUAU	3
osa-miRf11372-npr	UGUUACUCCCUCCGUUCCAAAUUA	UGUUACUCCCUCCGUCCCAUAAUA	3
osa-miRf10810-npr	UACUACUCCCUCCGUUCUAAAUUA	UACUACUCCCUCCGUCCCAAUAUA	3
osa-miRf12040-npr	UACUACCUCCGUUCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	3
osa-miRf10429-npr	UACUCUCUCCGUUUCAAAAUGUAA	UACUUCCUCCGUUUCAUAGUGUAA	3
osa-miRf11316-npr	AGCUACUCCCUCCGUUCCUUAAUA	GGUACUCCCUCCGUUCCUAAAUA	3
osa-miRf11808-npr	GUACUCCCUCCGUAUCAAAAUAUA	GUACUCCCUCCGUCCCUAAAUAUA	3
bdi-miR528	GUGGAAGGGGCAUGCAGAGGAGCA	UGGAAGGGGCAUGCAGAGGAG	3
osa-miR528-5p	GUGGAAGGGGCAUGCAGAGGAGCA	UGGAAGGGGCAUGCAGAGGAG	3
sbi-miR528	GUGGAAGGGGCAUGCAGAGGAGCA	UGGAAGGGGCAUGCAGAGGAG	3
zma-miR528a-5p	GUGGAAGGGGCAUGCAGAGGAGCA	UGGAAGGGGCAUGCAGAGGAG	3
zma-miR528b-5p	GUGGAAGGGGCAUGCAGAGGAGCA	UGGAAGGGGCAUGCAGAGGAG	3
osa-miRf10429-npr	UACUCCCUCCGUUCCAUAAUGUAA	UACUUCCUCCGUUUCAUAGUGUAA	3
osa-miRf12039-npr	AGAUACUCCCUCCGUUCCAUAAUU	AGGUACUCCCUCCGUCCCAUAAUAU	3
osa-miRf10429-npr	UACUCCCUCCGUUUCAUAUUAUAA	UACUUCCUCCGUUUCAUAGUGUAA	3
osa-miRf11604-npr	UACUCUCUCCGUCUCAAAAUAUAA	CUACUCUCUCCGUCCCAAAAUAAA	3
osa-miRf11603-npr	UAUUCCCUCCGUCCCAAAAUAAUU	CUACUCCCUCCGUCCCAAAAUAAGU	3
stu-miR7996a	AUGUGGAAAAUAUGAAUUUUGAAC	AUGUGGUACAUAUGAAAUUUGAAA	4
stu-miR7996b	AUGUGGAAAAUAUGAAUUUUGAAC	AUGUGGUACAUAUGAAAUUUGAAA	4
stu-miR7996c	AUGUGGAAAAUAUGAAUUUUGAAC	AUGUGGUACAUAUGAAAUUUGAAA	4
osa-miRf10218-npr	UAUACUCCCUCCGAUUCAUAAAAA	UAUACUCCCUCCGUCCCAAAAAA	4
osa-miRf10179-npr	AUCUACUUCCUCUGUUCCACAAUA	UACUACUUCCUCCGUCCCAAAAUA	4
tae-miR1130	CCUCCGUUCCUUAAUAUAAGACGU	CCUCCGUCUCGUAAUGUAAGACG	4
osa-miRf11002-npr	CCUCCGUCCCACGAAAGUUAUCUA	CCUCCGUCCCAAAAUGUAGCUA	4
osa-miRf11372-npr	UGUACUCCCUCCGUUCCACAAUAU	UGUUACUCCCUCCGUCCCAUAAUA	4
osa-miRf11808-npr	GUACUCCCUCCGUUCUAAAUUAGU	GUACUCCCUCCGUCCCUAAAUAU	4
osa-miRf10742-npr	AAAUACUACUCCCUCCGUUCCAUA	AAUGACUACUCCCUCCGUCCCAAA	4
sbi-miR1432	UGUGCUCAGGAGAGAUGACACCGA	CUCAGGAGAGAUGACACCGA	4
sit-miR115-npr	UGUGCUCAGGAGAGAUGACACCGA	CUCAGGAGAGAUGACACCGA	4
zma-miR1432-5p	UGUGCUCAGGAGAGAUGACACCGA	CUCAGGAGAGAUGACACCGA	4
osa-miRf10429-npr	UACUCCCUCCGUAUCAAAAUGUAA	UACUUCCUCCGUUUCAUAGUGUAA	4
osa-miRf10180-npr	AGUACUUCCUCUGUUCCAAAAUAU	ACUACUUCCUCCGUCCCAAGAUAU	4
osa-miRf12040-npr	UGCUCCCUCCGUUCCAUAUUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	4
osa-miRf11372-npr	UGUACUCCCUCCGUUCCAUAUUAU	UGUUACUCCCUCCGUCCCAUAAUA	4
bdi-miR5174d-3p	CAUCUAUUUUGGAACGGAGGGAGU	CAAUCUUUAUGGAACGGAGAGAGU	4

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Table 10. (cont.)

tae-miR2001	CGCUCGCCGGCAGCUACGGUGUCA	CGCUCGCCGGAGCAGCGUGCA	4
osa-miR5522	AACAGUAGUCGACAUGGGAGGCAU	AACAAUAGGAAUGGGAGGCAU	4
mtr-miR5298b	GAUGAGAGAUGGUAAGAAGAUGAA	UGAUGGAGAUGAUAUGAAGAUGAA	4
mtr-miR5298c	GAUGAGAGAUGGUAAGAAGAUGAA	UGAUGGAGAUGAUAUGAAGAUGAA	4
osa-miRf10429-npr	UACUUCCUCUGUUUCGAAAUGUAA	UACUUCCUCCGUUUCAUAGUGUAA	4
osa-miRf10571-npr	UUAUAUACUCCCUCCGUCCUAAAA	AUAUUUACUCCCUCCGUCCCAUAA	4
osa-miRf11612-npr	UACUACCUCCGUACCAAAAUAUAA	UACUCCCUCCGUCCCUAAAUAUA	4
osa-miRf10571-npr	CUAUAUACUCCCUCCGUUCCAUAU	AUAUUUACUCCCUCCGUCCCAUAA	4
osa-miRf11603-npr	UACUCCCUCCGUCCUAAAAAAGAU	CUACUCCCUCCGUCCCAAAAUAAGU	4
osa-miRf11946-npr	CCCGGUUCGUGAUACGAACUGGGA	CCCGGUUGGUGUUACCAACCGGGA	4
osa-miRf11524-npr	GGGAACCUUUAGUCCCGGUUCGUG	GGGAUCUUUAGUCCCGGUUGGUA	4
osa-miRf10735-npr	UACUACUCCCUCCGAUCCAUAAAA	UAGUACUCCCUCCGUCCCUAAAUA	4
osa-miR6248	UUUGAGAUGGAGGUAGUAG	UAUUUGAGGAUGGAGGUAGUA	4
osa-miRf11808-npr	GUACUCCCUGUGUCUCAUAAUAUA	GUACUCCCUCCGUCCCUAAAUAUA	4
osa-miRf12040-npr	UACUCCCUGUGUCUCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	4
osa-miRf10735-npr	UGUAUUCCCUCCGUUUCUAAAUAU	UAGUACUCCCUCCGUCCCUAAAUAU	4
osa-miRf11182-npr	GAUACUCCCUCCGUCCGUGAAUAA	GUACUCCCUCCGUCCCAUGAAAAA	4
osa-miRf11612-npr	UACUCCCUCCGUCCAUAAAAAUUC	UACUCCCUCCGUCCCUAAAUAUA	4
osa-miRf12040-npr	UACUCCCUCCGUCCCUUUUAUAAG	ACUCCCUCCGUCCCAUAAUAUAAG	4
osa-miRf10321-npr	UAUUACUCGCUCCGUCUAAAAAUA	UUAUACUCCCUCCGUCCCAAAAUA	4
osa-miRf11952-npr	UUACUCCCUCCGUUCCAUUUUAGA	UUACUCCCUCCGUCCCAAUAUAUA	4
osa-miRf11316-npr	GCUACUCCCUCCGUUCUAAAUUAG	GGUACUCCCUCCGUUCCUAAAUA	4
osa-miRf11316-npr	GCUACUCCCUCCGUUCUAAAUUAG	GGUACUCCCUCCGUUCCUAAAUA	4
osa-miRf10735-npr	UAGCUACUCCCUCCGUUCUAAAUU	UAGUACUCCCUCCGUCCCUAAAUA	4
osa-miRf11954-npr	UAUUAGCUACUCCCUCCGUUCUAA	UUUAGUACUCCCUCCGUCCCAA	4
osa-miRf12040-npr	UACUACCUCUGUUCCAUAAUAUAA	ACUCCCUCCGUCCCAUAAUAUAA	4
osa-miRf11612-npr	UACUCCCUCCGAUCCAUAAAAAAU	UACUCCCUCCGUCCCUAAAUAUA	4
osa-miRf11060-npr	UAUUUAGGAACGGAGGGAGUAGCU	UAUCAUGGGACGGAGGGAGUACU	4
osa-miRf11762-npr	AACCCUUUAGUACCGGUUGGUCUU	AAUCUUUAGUCCCGGUUGGUGUU	4
osa-miRf10429-npr	UACUACCUCCGUUCCAUAAUGUAU	UACUUCCUCCGUUUCAUAGUGUAA	4
gma-miR159c	CUUGGAUGGUGAAGGAGAGCUCCG	AUUGGAGUGAAGGGAGCUCCG	4
osa-miRf11603-npr	CUACUCCCUCCGUUCUCUAAAAAG	CUACUCCCUCCGUCCCAAAAUAAG	4
osa-miRf12035-npr	UAUACUACUCCCUCCGAUCCAUAA	UGAACUACUCCCUCCGUCCCAUAA	4
osa-miRf11612-npr	UACUCCCUCCGAUCCAUAAAAAAU	UACUCCCUCCGUCCCUAAAUAUA	4
osa-miRf11182-npr	GUACUCCCUCCGUUCCAUAUAAAU	GUACUCCCUCCGUCCCAUGAAAAA	4
osa-miRf12040-npr	ACUCCCUCCGUUCCAUAUAAAUUG	ACUCCCUCCGUCCCAUAAUAUAAG	4
osa-miR396f-5p	AUGCUUUCCACAGGCUUUCUUGAA	UCUCCACAGGCUUUCUUGAA	4
osa-miRf10429-npr	UACUACCUCUGUUUCAUAAUAUAA	UACUUCCUCCGUUUCAUAGUGUAA	4
osa-miRf10429-npr	UACUACCUCUGUUUCAUAAUAUAA	UACUUCCUCCGUUUCAUAGUGUAA	4
sbi-miR6220-5p	CUCCAUUCCUAAAUAUAAGACGUC	CUCCAUCCUAAAUUAUAAGACAUU	4
osa-miRf11808-npr	GUACUACCUCUGUUCCAUAAUAUA	GUACUCCCUCCGUCCCUAAAUAUA	4

Source (see APPENDIX B) and target (see APPENDIX C) based miRNA and their differential expression results between control and stress samples were identified based on transcriptome data. The miRNAs that were found in both target and source based tables are shown in Table 11. A total of 23 different miRNA (10 of them uncharacterized) with 25 different target (8 of them uncharacterized) were identified from the Puccinellia transcriptome and their miRNA and target sources were differentially regulated. pdi-mir-

7139 was found to be upregulated based on its occurrence in Puccinellia boron treated samples (FPKM: 6.30). However its target transcript was downregulated (FPKM: -1.51). pdi-mir-7139 was uncharacterized, but its target was annotated as a leucine-rich repeat kinase. pdi-mir-10551 was annotated as a probable mitochondrial adenine nucleotide transporter and it was upregulated (FPKM: 3.28). Its target was identified as cytochrome c oxidase subunit (FPKM: 19.48). pdi-mir-8388 was a potassium channel and was upregulated (FPKM: 1.24), while its target was annotated as 3-ketoacyl synthase (FPKM: -2.73). pdi-mir-6499 and pdi-mir-20763 were slightly upregulated in stressed samples (FPKMs: 0.48) and annotations were not known, whereas their targets which were involved with the photosystem II reaction center were highly downregulated (FPKMs: -68.51). pdi-mir-19561 was sourced from beta-galactosidase transcript and it was slightly upregulated (FPKM: 0.30). This miRNA was targeted to the transcription initiation factor IIE subunit which was found to be upregulated under boron stress (FPKM: 1.27). Two of the identified miRNAs (pdi-mir-20326 and pdi-mir-11297) were found as self-regulated, their differential expression levels were the same for the miRNA and target sources (FPKM: -8.81 and -65.59, respectively).

Table 11. MiRNAs for which differential expression was observed in both source and target in transcriptome data

mirnaName	mirnaSourceAccession	Target_Acc.	mirna Source Description	Target Description	Difference between control vs stress FPKM (mirna source)	Difference between control vs stress FPKM (target)
pdi-mir-4085	TRINITY_DN190951_c1_g1_i1	TRINITY_DN147052_c0_g1_i1	UBC30_ARATHUbiquitin-conjugating enzyme E2 30 OS=Arabidopsis thaliana GN=UBC30 PE=2 SV=1	---NA---	1.49	0.67
pdi-mir-4085	TRINITY_DN190951_c1_g1_i1	TRINITY_DN147052_c0_g2_i1	UBC30_ARATHUbiquitin-conjugating enzyme E2 30 OS=Arabidopsis thaliana GN=UBC30 PE=2 SV=1	F4J8G2_ARATHReceptor like 33 OS=Arabidopsis thaliana GN=RLP33 PE=4 SV=1	1.49	0.82
pdi-mir-6499	TRINITY_DN136444_c0_g1_i1	TRINITY_DN189280_c0_g1_i1	OS=Oryza sativa GN= PE=4 SV=1	PSBK_ORYSAPhotosystem II reaction center K OS=Oryza sativa GN=psbK PE=3 SV=1	0.48	-68.51
pdi-mir-7139	TRINITY_DN214173_c4_g2_i1	TRINITY_DN187876_c0_g1_i4	OS=Oryza sativa GN= PE=4 SV=1	Q9SN80_ARATHLeucine-rich repeat kinase family OS=Arabidopsis thaliana GN= PE=2 SV=1	6.30	-1.51
pdi-mir-8388	TRINITY_DN164731_c0_g1_i1	TRINITY_DN156213_c0_g1_i1	GORK_ARATHPotassium channel GORK OS=Arabidopsis thaliana GN=GORK PE=1 SV=2	AEGTA M8CUR0 3-ketoacyl-synthase 12 OS=Aegilops tauschii GN=F775_22063 PE=4 SV=1	1.24	-2.73
pdi-mir-9912	TRINITY_DN294611_c0_g1_i1	TRINITY_DN178052_c1_g2_i1	---NA---	---NA---	1.07	1.66
pdi-mir-10155	TRINITY_DN146224_c0_g1_i1	TRINITY_DN224249_c0_g7_i7	---NA---	NMNAT_ARATHNicotinamide nicotinic acid mononucleotide adenylyltransferase OS=Arabidopsis thaliana GN=NMNAT PE=2 SV=1	7.60	6.83
pdi-mir-10551	TRINITY_DN205990_c2_g2_i2	TRINITY_DN284184_c0_g1_i1	BRTL3_ARATH Probable mitochondrial adenine nucleotide transporter BTL3 OS=Arabidopsis thaliana GN=At5g64970 PE=2 SV=1	COX2_ARATHCytochrome c oxidase subunit 2 OS=Arabidopsis thaliana GN=COX2 PE=1 SV=2	3.28	19.48

(Cont. on the next page)

Table 11. (cont.)

pdi-mir-10626	TRINITY_DN168068_c0_g2_i1	TRINITY_DN179579_c0_g1_i3	---NA---	SUI12_ARATH translation factor SUI1 homolog 2 OS=Arabidopsis thaliana GN=At1g54290 PE=3 SV=1	1.05	1.17
pdi-mir-11297	**TRINITY_DN218940_c4_g13_i1**	**TRINITY_DN218940_c4_g13_i1**	**---NA---**	**---NA---**	-65.59	-65.59
pdi-mir-12004	TRINITY_DN99661_c0_g1_i1	TRINITY_DN119910_c0_g1_i1	NU3M_ARATHNADH-ubiquinone oxidoreductase chain 3 OS=Arabidopsis thaliana GN=ND3 PE=2 SV=2	NU3M_ARATHNADH-ubiquinone oxidoreductase chain 3 OS=Arabidopsis thaliana GN=ND3 PE=2 SV=2	13.62	15.63
pdi-mir-14004	TRINITY_DN157066_c0_g1_i1	TRINITY_DN207025_c5_g1_i1	---NA---	---NA---	1.43	8.52
pdi-mir-14464	TRINITY_DN200410_c0_g3_i1	TRINITY_DN167034_c0_g1_i1	CSK2P_ARATHCasein kinase II subunit alpha- chloroplastic OS=Arabidopsis thaliana GN=CKA4 PE=2 SV=1	A0A0U2JSE5_ORYSANBS-LRR-like resistance OS=Oryza sativa GN=Os01g02250 PE=4 SV=1	1.18	1.99
pdi-mir-16111	TRINITY_DN214853_c4_g2_i5	TRINITY_DN254674_c0_g1_i1	Q9XI10_ARATHDPP6 N-terminal domain OS=Arabidopsis thaliana GN= PE=2 SV=1	ODPB1_ARATHPyruvate dehydrogenase E1 component subunit beta- mitochondrial OS=Arabidopsis thaliana GN=PDH2 PE=1 SV=2	2.67	1.04
pdi-mir-18173	TRINITY_DN215466_c1_g1_i1	TRINITY_DN200970_c0_g2_i1	---NA---	9ORYZ Q1KL68 Lysine ketoglutarate reductase saccharopine dehydrogenase biofunctional enzyme OS=Zizania latifolia GN=LKR SDH PE=2 SV=1	0.67	3.49
pdi-mir-19561	TRINITY_DN215903_c3_g5_i2	TRINITY_DN2019_c0_g1_i1	BGAL6_ARATHBeta-galactosidase 6 OS=Arabidopsis thaliana GN=BGAL6 PE=2 SV=1	WHEAT W5E476 Transcription initiation factor IIE subunit beta OS=Triticum aestivum PE=3 SV=1	0.30	1.27

(Cont. on the next page)

Table 11. (cont.)

pdi-mir-20110	TRINITY_DN165558_c0_g1_i1	TRINITY_DN197630_c0_g1_i1	F4KEF0_ARATHLeucine-rich repeat kinase OS=Arabidopsis thaliana GN=At5g39390 PE=3 SV=1	RK2_ARATH50S ribosomal chloroplastic OS=Arabidopsis thaliana GN=rpl2-A PE=3 SV=1	1.06	-2.45
pdi-mir-20326	**TRINITY_DN211912_c6_g5_i2**	**TRINITY_DN211912_c6_g5_i2**	**---NA---**	**---NA---**	-8.81	-8.81
pdi-mir-20763	TRINITY_DN136444_c0_g1_i1	TRINITY_DN189280_c0_g1_i1	OS=Oryza sativa GN= PE=4 SV=1	PSBK_ORYSAPhotosystem II reaction center K OS=Oryza sativa GN=psbK PE=3 SV=1	0.48	-68.51
pdi-mir-21192	TRINITY_DN169019_c0_g1_i1	TRINITY_DN219386_c2_g2_i1	SOYBN I1LXS3 Uncharacterized protein OS=Glycine max PE=4 SV=1	---NA---	4.63	0.90
pdi-mir-21590	TRINITY_DN2797_c0_g1_i1	TRINITY_DN165445_c0_g2_i1	NDUA9_ARATHNADH dehydrogenase	OE64C_ARATHOuter envelope chloroplastic OS=Arabidopsis thaliana GN=OEP64 PE=1 SV=1	1.37	1.05
pdi-mir-22508	TRINITY_DN224438_c3_g3_i1	TRINITY_DN160101_c1_g1_i1	---NA---	ORYSI Q6W7J3 opsin OS=Oryza sativa indica PE=2 SV=1	-13.25	-6.00
pdi-mir-22562	TRINITY_DN203863_c3_g1_i10	TRINITY_DN198051_c2_g1_i1	Q8LCW1_ARATHAspartyl protease family OS=Arabidopsis thaliana GN=At4g33490 PE=2 SV=1	---NA---	0.96	0.88
pdi-mir-22562	TRINITY_DN203863_c3_g1_i10	TRINITY_DN198051_c2_g1_i2	Q8LCW1_ARATHAspartyl protease family OS=Arabidopsis thaliana GN=At4g33490 PE=2 SV=1	---NA---	0.96	0.83
pdi-mir-24320	TRINITY_DN195472_c2_g2_i1	TRINITY_DN175970_c0_g1_i1	F4IBK7_ARATHP-loop containing nucleoside triphosphate hydrolases superfamily OS=Arabidopsis thaliana GN=At1g65810 PE=4 SV=1	LAC6_ARATHLaccase-6 OS=Arabidopsis thaliana GN=LAC6 PE=2 SV=1	6.03	1.75

Bold ones indicate self-regulated miRNAs.

3.10. qRT-PCR Results of miRNA Analysis

Ten miRNAs were selected to validate the bioinformatics results (Figure 6). Since these miRNAs were selected from mature miRNAs, their presence and up- or down-regulation were observed with qRT-PCR. As a result of qRT-PCR, three of the selected miRNAs were found to be upregulated (miRf10218, miRf10571 and miRf12040). The remaining miRNAs were downregulated. Upregulated miRf10218 was annotated as a calcium/calmodulin dependent protein kinase which has many metabolic roles, such as signaling. A significant decrease was observed for osa-miRf10571-1. This miRNA was annotated as a phytosulfokine receptor with serine/threonine-protein kinase activity.

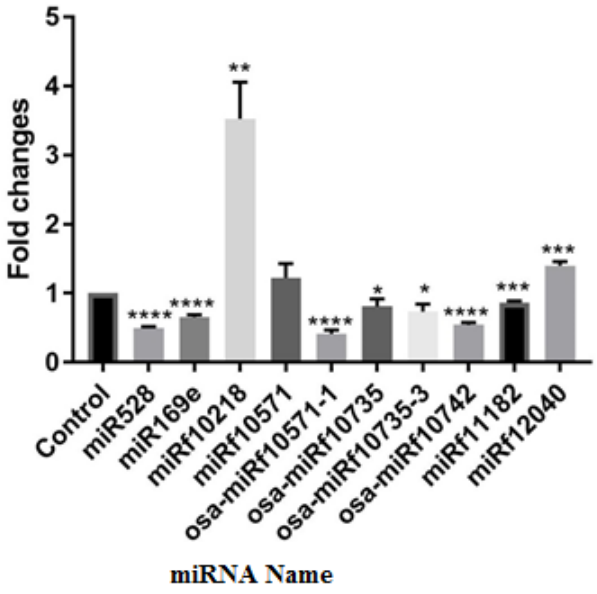

Figure 6. Real-time PCR results of *P. distans* transcriptome for ten selected transcripts. Actin was used as internal control and error bars indicate standard deviation of three technical repeats. **** indicates significant difference at $p \leq 0.0001$, *** indicates significant difference at $p \leq 0.001$, ** indicates significant difference at $p \leq 0.01$ and * indicates significant difference at $p \leq 0.05$ with respect to control.

CHAPTER 4

DISCUSSIONS

Phytoremediation and phytoextraction are clean, simple, cost-effective, environmentally friendly methods for removing toxic elements from soil [44]. These methods rely on plants such as *P. distans*, an extreme hyperaccumulator of boron. Elucidation of the mechanism(s) by which *P. distans* accumulates and tolerates normally toxic levels of boron may allow development of a faster growing, higher biomass boron hyperaccumulator. This study provides the first broad examination of *P. distans*' transcriptomic response to boron hyperaccumulation. According to the analysis of RNA-Seq data generated in this study, 3312 differentially expressed transcripts were identified and 1652 of them were annotated to 1107 unique proteins with homologs in *A. thaliana* and *O. sativa*. In contrast, 1660 transcripts had no homologs in *A. thaliana* or *O. sativa*. Thus, these transcripts may be specific to *P. distans* and some may play roles in boron hyperaccumulation, a mechanism which is not present in *A. thaliana* and *O. sativa*.

Plants activate functional and regulatory genes to avoid or tolerate disruptive situations that accompany environmental stresses such as drought, cold, salinity and metalloid accumulation [113-114]. Many of these genes play roles in basic plant metabolism and are not specific to the stress response. Therefore, this discussion will mainly focus on stress-related proteins, transporters and plant hormones that are differentially regulated under excess boron.

Stress-related molecules

Stress-related molecules are known to have roles in the plant's response to a wide variety of biotic and abiotic conditions [115]. According to KEGG analysis, certain stress-related molecules were highly active under excess boron as compared to normal boron levels. For example, the *A. thaliana* homolog of UDP-glycosyltransferase (UniProtID: U88A1_ARATH; Transcript Name: TRINITY_DN223147_c5_g2_i1:16-443), which plays a role in flavonoid biosynthesis, was highly up-regulated (see APPENDIX D). Flavonoids are well-known antioxidants and are important in detoxifying the excess free radicals that plants produce under stress.

The ubiquitin/26S proteasome pathway is important for protein degradation and controls protein level and activity. In general, 26S proteasome activity is induced under stress conditions. For example, its activity was calculated as 11.46 fold higher under boron stress in *Gypsophila perfoliata*, a boron hyperaccumulator plant [116]. Similarly, 26S proteasome and its subunits were upregulated under excess boron in the present study. A possible explanation is that the 26S proteasome machinery is important to degrade proteins damaged as a result of toxicity.

Another pathway that was affected by high boron stress was the malate pathway. Activation of this pathway is correlated with abiotic stress [117]. Four malate-related *A. thaliana* homolog proteins (UniProtIDs: B3H477_ARATH, FUM1_ARATH, FUM2_ARATH, and MAOP3_ARATH) were down-regulated under excess boron. In contrast, malate dehydrogenase 1 enzyme, which is responsible for converting malate to oxaloacetate, was up-regulated (UniProtID: MDHC1_ARATH) (see APPENDIX D). This combined increase in malate dehydrogenase 1 and decreased expression of two fumarate hydratases (UniProtIDs: FUM1-2 and B3H477) under boron stress indicates altered regulation in the glyoxylate pathway at malate (Figure 7a) which is in line with previous findings [118]. In this pathway, isocitrate is converted to malate via the intermediate molecule, glyoxylate. Glyoxylate and pyruvate can also be formed by a low affinity reaction catalyzed by 4-hydroxy-4-methyl-2-oxoglutarate aldolase (Figure 7b) (Maruyama, 1990). In boron-stressed plants, two 4-hydroxy-4-methyl-2-oxoglutarate aldolases (RRAA2_ARATH and RRAA3_ARATH) were significantly up-regulated (FPKMs: 426.28 and 253.61, respectively) (see APPENDIX D). Thus, high activity of these aldolases can result in increased glyoxylate which is then converted to malate. Malate can then be transported out of the cell with ions like K+ in order to balance cellular pH, a mechanism that is known to be a response to aluminum tolerance in wheat [119]. Thus, these results indicate that malate and related proteins have critical roles in boron hyperaccumulation.

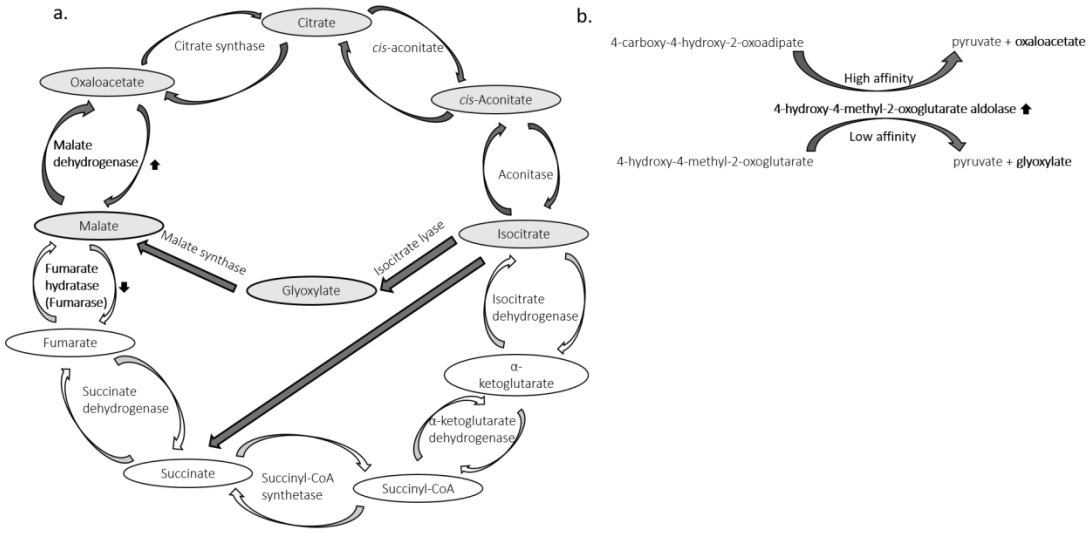

Figure 7. The interaction of 4-hydroxy-4-methyl-2-oxoglutarate aldolase with glyoxalate pathway a) The glyoxalate pathway and b) 4-hydroxy-4-methyl-2-oxoglutarate aldolase reactions. Molecules in bold font were affected by boron stress.

Plant cells are enclosed within cell walls which form physical barriers and provide tolerance to turgor pressure [120]. The cell wall consists of polysaccharides, proteins and phenolic compounds [121]. Boron is another important structural component of the cell wall as it crosslinks rhamnogalacturonan-II molecules, thereby providing stability. Expansin proteins are also found in the wall and are responsible for cell enlargement by loosening the wall [122]. Expansins directly interact with RD22 proteins to mediate cell enlargement [123]. RD22 proteins contain a BURP domain, an amino acid sequences which is highly conserved in plant species [124]. As an acronym, BURP comes from: BNM2 (*Brassica napus* microspore-derived embryos protein) [125], USP (*Vicia faba*; unidentified seed protein) [126], RD22 (*A. thaliana* protein responsive to desiccation) [127], and PG1β (*Solanum lycopersicum* β subunit of polygalacturonase isozyme 1) [128]. Here we detected the upregulation of the *A. thaliana* homolog of the RD22 protein (Table 3). In addition to its role in cell enlargement, RD22 was reported to be responsible for increased levels of lignin precursor in cell walls under salt stress [123]. Under high boron, the excess boron has been shown to interact with lignin precursors in *Pinus radiata* organ cultures [7] and chitin-binding lectin precursors in barley leaves [129]. Therefore, the up-regulated RD22 homolog in *P. distans* may provide additional lignin precursors, thereby increasing the boron binding capacity of cell walls under stress. Thus, RD22 may be an important component of the hyperaccumulation mechanism in *P. distans*. Similarly, BURP domain activity was also upregulated in rice under boron toxicity [130].

Transporter proteins

The first identified boron tolerance-related gene was *Bot1* which expresses a putative transmembrane B efflux transporter in barley [7]. Many studies on boron efflux transporters showed that the presence of these transporters is controlled by cellular boron concentration. Miwa et al. (2007) [131] observed that under low boron concentrations, AtBOR1 facilitates boron uptake from roots to shoots; while under high boron, AtBOR1 undergoes endocytosis [131-132]. These findings indicate that boron concentration affects the dynamics of transporter proteins. A possible explanation is that boron itself interacts with the ribose in mRNA and changes its stability thereby affecting transcription and translation [133]. In our study, the *A. thaliana* BOR6 homolog (UniProtID: BOR6_ARATH), a transporter for boron efflux, was up-regulated in *P. distans* (Table 5). Since *P. distans* is a hyperaccumulator plant, potential roles of this boron transporter in the cell are: *i)* facilitation of boron transport from root to shoot and *ii)* transport of boron from the cell into the cell wall. On the other hand, Ramila et al. (2016) found that *BoT1* transcripts increased under excess boron in Puccinellia [45]. The *BoT1* primers from that study [45] were also used during validation of the bioinformatic analysis of the present study. Based on the analysis, no significant increase was observed with quantitative real-time PCR (Figure 3). This result was consistent with a previous study in barley [134]. Tombuloglu et al. (2015) suggested that the *Bot1* gene has seven duplicated paralogs and their expression was not dependent on boron concentration in barley [134].

Major intrinsic proteins (MIPs), also known as aquaporin proteins, facilitate the transport of water across biological membranes and are permeable to other small, uncharged molecules such as glycerol, solutes and ions [133, 135-137]. The transportation capacity/selectivity of aquaporins depend on channel pore size [138]. MIPs are categorized into seven subgroups in the plant kingdom including NOD26-like intrinsic proteins (NIPs) and tonoplast intrinsic proteins (TIPs) transporters [139-140]. NIPs are generally located in the plasma membrane and endoplasmic reticulum; TIPs are localized in the vacuole membrane (tonoplast) and thylakoid inner membrane [139]. NIPs are the most divergent plant MIP subfamily with respect to their substrate specificities and amino acid sequences. The NIP aquaporin family is unique to plants and NIPs are selectively permeable to metalloids [133]. Specifically, NIP II and NIP III actively transport boron, while Hv*NIP2*;1 had reduced expression in barley under excess boron [141-142]. NIP proteins were also grouped among them according to sequence similarities, such as NIP1:1, 2:1 and 2:2 [138] and their expression levels differed under excessive boron in barley. In our

study, two types of aquaporins were identified, one of them was a homolog of an *O. sativa* NIP (UniProtID: Q949A7_ORYSA; Transcript Name: TRINITY_DN210823_c0_g3_i1:4-269) and the other was a homolog of an *A. thaliana* TIP (UniProtID: TIP13_ARATH; Transcript Name: TRINITY_DN222891_c3_g1_i1:0-216) (Table 5). While Yildirim and Uylas (2016) identified that NIP transcripts were downregulated under boron stress in poplar [143], the *O. sativa* homolog NIP-type aquaporin was up-regulated in *P. distans* under boron stress. Overexpression of an aquaporin could cause tolerance to distinct stress conditions. For example, PgTIP1 was overexpressed in *A. thaliana* and plants showed increased tolerance to salt and drought stress but decreased tolerance to cold [144]. However, Pang et al. (2010) identified AtTIP5;1 in Arabidopsis and they observed downregulated expression level [145], in contrast with our results. Therefore, our results suggest that the *O. sativa* NIP aquaporin is responsible for cellular boron uptake in *P. distans*. On the other hand, the *A. thaliana* TIP homolog was down-regulated indicating that not all aquaporins are involved in boron transport and/or that the vacuole is not an important storage site for boron. Although the bidirectional activity of PIPs (plasma membrane intrinsic proteins) was demonstrated in rice under boron toxicity [146-147], none of these transcripts were identified in the present study.

ABC transporters are members of a diverse family. Their substrate specificities vary from carbohydrates to ions and some of them are completely specialized to transport ions and solutes. For example, mntABCD is an ABC transporter and selectively permeable for manganese [148]. In our study, five different *A. thaliana* ABC transporters (ABC-B, C, E, F, I) were differentially expressed under boron stress. All of the ABC transporters were up-regulated with the exception of AB17I_ARATH. The activation of this transporter group may be due to the fact that: *i)* ABC transporters are permeable to a wide variety substrates and some of them may be able to transport boron and *ii)* they can transport boron-binding molecules such as sorbitol. Thus, ABC transporters may be a significant component of the boron hyperaccumulation mechanism in *P. distans*.

Under excess boron, a sulfate transporter was down-regulated in *P. distans* (UniProtID: Q8S317_ORYSA). Sulfate transport mechanisms are divided into four groups. The first is sulfate co-transport with a proton, the second is co-transport with sodium (Na), and the third is sulfate antiport with an anion. The fourth mechanism is ATP-dependent ABC transporter-mediated sulfate uptake [149]. Sulfate transporters play important roles in response to metal stresses in a metal-specific manner [150]. Sulfate transporters are responsible for transport of some oxyanions, such as molybdate and

chromate [150-152] observed that seven sulfate transporters were up-regulated by arsenate and cadmium, ten were up-regulated by chromium and five by lead exposure. However, our study indicated down-regulation of a sulfate transporter in response to boron hyperaccumulation. Because excess boric acid will acidify the cellular environment, less proton pumping is required to maintain cellular pH.

Depending on plant type, sugars (such as sucrose), sugar alcohols (such as mannitol) and amino acids (such as glycine) accumulate under stress conditions and these help plants cope with stress [153]. Sugar alcohols provide protection from stress by scavenging hydroxyl radicals and/or stabilizing macromolecular structure [154]. Inositol is a sugar alcohol which also has a role in the biogenesis of the uronosyl and pentosyl units of pectin, hemicelluloses, and related structures in plant cell walls [155]. An inositol transporter was up-regulated in *P. distans* (UniProtID: INT2_ARATH; Transcript name: TRINITY_DN172179_c0_g2_i1:0-695,2.5518) under high boron. An increased level of this transporter could be used in the cell to increase the level of inositol available to scavenge reactive oxygen species and/or chelating boron, thereby protecting the cell. Increased cellular inositol may also be required for the synthesis of cell wall pectin and its precursor to bind to the increased amount of boron in the cell wall. The similarity between the inositol and boron transporters of *A. thaliana* [8] suggests a third possibility: the inositol tranporter may also transport boron.

Transporter proteins were also played important roles to cope with boron stress in boron hyperaccumulator plants, poplar and Puccinellia [43, 45, 143]. While BOR1 and NIP channel proteins were downregulated in poplar under excess boron [143], the expression of BOR1 transporter protein in Puccinellia was higher in roots than shoots [45]. Consistently with Ramila et al. (2016), the NIP channel and boron exporter transporter proteins were found as upregulated in our study. Additionally, Kato et al. (2008) was suggested the BOR1 and NIP5;1 enhancement is an effective strategy to improve boron flow [156]. Not only boron transporter proteins but also ABC-type transporter proteins were regulated in excess boron in this study and previous studies [43, 45]. Ramila et al. (2016) suggested that the possible explanation of increasing ABC-type of transporters was involving in detoxification and protection of plant from harmful effect of boron stress [45].

Plant hormones

Plant hormones play critical roles in stress conditions by providing physiological and biochemical responses to stress factors [157]. Abscisic acid (ABA) signaling is the central regulator of the abiotic stress resistance pathway in plants [158]. Under drought

conditions, plants cope with stress by increasing the amount of ABA causing stomatal closure and reduced transpiration [159]. In our dataset, an *A. thaliana* homolog of ABA and related proteins such as phosphatase (UniProtID: P2C16_ARATH; Transcript Name: TRINITY_DN220107_c1_g6_i1:12-396) was down-regulated (see APPENDIX D). The absence of ABA is associated with increases in cytosolic free calcium, calcium-dependent proteins [160] and reactive oxygen species [161]. According to our findings, calcium-dependent proteins were up-regulated under excess boron, thus, supporting the relationship between ABA and calcium-related proteins. In addition, it is well-known that both calcium and boron are important components of pectic polysaccharides in the cell wall [162]. Therefore, an increase in boron could trigger up-regulation of calcium levels and calcium-dependent proteins in order to maintain cell wall integrity. Ethylene and ABA are antagonists: ethylene triggers catabolism of ABA [157]. In our analysis, five ethylene response proteins had increased expression under stress (see APPENDIX D). Thus as expected, the ethylene response was up-regulated while ABA was down-regulated under boron stress.

Another plant hormone that has a role in stress defense is gibberellin (GA). GAs are involved in cell wall loosening in plants, thus, contributing to cell expansion [163-164]. In addition, GAs play roles in plant development and growth, thus they are inhibited under stress conditions to limit growth [165]. For example, GA was reduced under high salinity stress in *A. thaliana* [166]. Interestingly, in our study, positive regulators of GAs (UniProtIDs: TSN1_ARATH, TSN2_ARATH; Transcript Names: TRINITY_DN151004, TRINITY_DN122991) had increased expression under boron stress (see APPENDIX D). The excess GAs produced under stress may maintain cell wall looseness as it is known that boron toxicity is usually accompanied by increased cell wall rigidity. Thus, GAs may have a critical role in *P. distans* boron tolerance by preventing cell rigidity, thereby allowing survival and continued growth.

Other studies also identified signaling related proteins under excess boron [43, 45, 129, 143]. Calcium-related proteins were found to be increased in boron stress conditions [129, 143]. These results were consistent with the present study. Yildirim and Uylas (2016) suggested that calcium-related proteins could be involved with absorption and storage of excess boron in the cell [143].

MiRNAs of Puccinellia

Elucidation of boron hyperaccumulation mechanisms is important for understanding both phytoremediation and tolerance approaches. Recent studies indicate

that not only tolerance genes but also small RNAs, such as miRNAs, are important to regulate gene expression and play important roles in adaptation [81]. In the present study, sequenced RNA samples were used to construct a reference genome. Potential miRNAs from this reference genome were predicted based on their potential hairpin structure which were filtered based on their length value which was set as minimum 30. A total of 24,657 sequences were extracted as potential miRNAs from Puccinellia. These sequences were used to predict mature miRNAs which have close relationships to known miRNAs. Additionally, predicted miRNAs were then searched in transcriptome and proteome datasets to understand whether they possibly have roles in boron hyperaccumulation and/or stress.

Predicted mature miRNAs which are conserved were identified under boron stress conditions in *P. distans*. mir169 is the most conserved miRNA family in plants and plays roles in abiotic and biotic stress conditions [167-169]. mir169 was upregulated in barley leaves under excess boron and targeted to a nuclear transcription factor [170]. Therefore, the presence of mir169 in *P. distans* possibly shows that despite this plant's ability to tolerate very high levels of boron, boron activates abiotic stress responses. Another miRNA family in *P. distans* is miRf10571/10571-1/12040. These miRNAs have roles as a phytosulfokine (PSK) receptor. PSK is a pentapeptide and its amino acid sequence is Tyr-Ile-Tyr-Thr-Gln with sulfate attached to each tyrosine residue [171]. Peptide signaling is known as an effector of cell-cell communication [172] and miRf10571/10571-1/12040 are possibly important for stress communication mechanisms to avoid the harmful effects of boron.

The plant immune system can recognize self and/or non-self and induce the plant defense system with resistance genes (R) to respond to non-self-proteins [173]. The NB-ARC domain itself is constructed from three subdomains: a nucleotide-binding (NB) domain and a C-terminal extension that forms a four-helix bundle (ARC1) and a winged-helix fold (ARC2). The NB-LRR proteins are members of NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) domain [174]. miRf10742 and miRf11182 were identified in boron stress conditions and are targeted to NB-ARC domain containing protein. These findings suggest that these two miRNAs have roles in boron tolerance mechanisms of *P. distans* by their interactions with R genes.

In many stress conditions, activation of the calcium/calmodulin mediated signal network was observed to be induced under environmental stimuli [175-176] and it is a response network against many biotic and abiotic stresses [177]. Calmodulin proteins and

calcium/calmodulin-dependent protein kinases activate several targets in cellular processes [175]. Under excess boron, more than one miRNA family was identified, such as miRf10735, osa-miRf10810-npr and osa-miRf11603-npr which have roles in calcium signaling. Excess boron triggers the calcium pathway to induce the whole plant defense and/or starts new signaling pathways which do not exist in non-hyperaccumulator plants and activates boron accumulation.

Conserved miRNAs from both source and target based transcriptome were identified in this study (Table 11). Seven conserved miRNAs with identified targets were identified as upregulated under boron stress whereas their targets were dramatically decreased. The annotation of pdi-mir-7139 was not identified while its target was a kinase family protein, leucine-rich repeat kinase family. The function of this type of kinase is to act as a transmembrane receptor and such proteins have roles in signaling pathways, and activating plant immune responses [178]. The possible role of this miRNA in boron hyperaccumulation is that by deactivating its target, pdi-mir-7139 inhibits the induction of the plant immune system. Another miRNA, pdi-mir-8388 originates from a potassium channel protein GORK and is targeted to 3-ketoacyl synthase. Wei et al. (2005) suggested that GORK protein is upregulated under cold stress to prevent cellular dehydration, while fatty acid biosynthesis was also upregulated [179]. In the present study, the predicted miRNA (pdi-mir-8388) source was upregulated and it is targeted to fatty acid biosynthesis by reducing its level.

Leucine-rich repeat proteins are affected both ways: by being target, their expression is decreased (pdi-mir-7139) and by being a miRNA source. Pdi-mir-20110 also originates from the leucine-rich repeat kinase family and is targeted to chloroplastic ribosome. Thus, if miRNA is cleaved from it, the protein cannot be functional. Thus, probably more than one signaling pathway is effective in hyperaccumulation mechanisms. The remaining four miRNAs are quite interesting. These are pdi-mir-6499, -20763, -20326 and -11297. The first two miRNAs are uncharacterized in terms of miRNA sources and even a small increase of their transcript level (FPKM: 0.48, both) was associated with down-regulation of their targets which are the same, the photosystem II reaction center (PSII) (FPKM: -68.51). Abiotic or biotic stress conditions result in an increase in reactive oxygen species (ROS) [180]. Salt stress down-regulated PSII and this situation enhanced ROS production [181]. Similarly, in our study PSII is highly down-regulated and it is possibly that the predicted miRNAs are responsible for this regulation.

In the present study, remaining two of miRNAs, pdi-mir-20326 and -11297, are expressed and this expression is targeted to their own transcripts, which means they are self-regulating miRNAs. Direct evidence about autoregulation of miRNAs was firstly shown for let-7 miRNA. The let-7 directly binds and regulates its own primary transcript [182]. Additionally, Castellano et al. (2009) suggested that miRNAs could act as a part of negative autoregulatory feedback loop [183]. Therefore, pdi-mir-20326 and -11297 are responsible to regulate their own transcripts in a negative manner. These miRNAs may have critical roles under boron stress as the possible presence of these transcripts could prevent excess boron uptake *i)* by inhibiting boron transport through cell, *ii)* and/or these transcripts have roles to accumulate metalloids in cell and Puccinellia chooses to eliminate it. By reducing their transcript level, plants could tolerate and/or hyperaccumulate boron. Since these miRNAs are uncharacterized, their functional and cellular roles should be identified to understand miRNA regulation of boron hyperaccumulation.

Boron efflux transporters are responsible from boron translocation from the cell and their activity changes with boron concentration. pdi-mir-24630 was a significant miRNA in terms of function because this miRNA originated from two different transcripts which were annotated as a Arabidopsis homologs of boron transporter BOR6. One of this miRNA's targets was annotated as a probable mediator of RNA polymerase II transcription subunit. Annotation of the other target could not be achieved, suggesting that it was a Puccinellia specific target. Under all conditions, pdi-mir-24630 was up-regulated (FPKM: 2.80 and 1.50). Another miRNA, pdi-mir-3738, was also up-regulated (FPKM: 2.50) under excess boron and its originated transcript could not annotated while its target was the Arabidopsis homolog of BOR6. The result of BOR6 regulation by miRNAs indicates that pdi-mir-3738 possibly regulates BOR6 in a positive manner. In our previous study, BOR6 itself was found to be upregulated. Thus, despite the origin of pdi-mir-24630 from this transcript, pdi-mir-3738 was capable of upregulating BOR6. Additionally, pdi-mir-24630 probably activated other genes which are responsible for boron accumulation and/or tolerance.

Major intrinsic proteins (MIPs), also known as aquaporin proteins, facilitate the transport of water across biological membranes. These type of transporters are also permeable to other small uncharged molecules (including gasses) such as glycerol, solutes and ions [133, 135-136]. MIPs were categorized into seven subgroups in the plant kingdom and one of them is tonoplast intrinsic proteins (TIPs). In our transcriptomics study, the

Arabidopsis homolog of TIP protein was found to be downregulated [184]. In the miRNA analysis, pdi-mir-19480 was annotated as originating from an Arabidopsis homolog of a TIP channel protein. The target of this miRNA was annotated as a 60S ribosomal protein. The upregulation of this miRNA (FPKM: 1.93) could indicate that excess boron possibly triggers the downregulation of TIP proteins. This may result in *i)* activation or deactivation of downstream genes to induce signaling, or *ii)* a decreasing TIP level suggesting that Puccinellia activates a new process to accumulate boron which is not seen in other boron tolerant plants.

CHAPTER 5

CONCLUSION

P. distans is a hyperaccumulator, monocot plant and is a non-model organism. Limited knowledge about this species and its hyperaccumulation mechanism led us to use a transcriptomics approach to elucidate stress tolerance and potential hyperaccumulation mechanisms in *P. distans*. Signaling and metabolic pathways in Puccinellia were previously studied under salinity, alkaline soil and chilling stress conditions [43, 92], therefore, we focused on stress-related molecules, transporters and plant hormones. Strong evidence was obtained that the boron tolerance and hyperaccumulation mechanism of *P. distans* involves alterations in the malate pathway, changes in cell wall components that allow sequestration of excess boron without toxic effects, and at least one putative boron transporter and two putative aquaporins. Transgenic overexpression of this boron transporter and aquaporins in *P. distans* or determination of their subcellular localization will provide deeper information about their direct role in the boron hyperaccumulation mechanism. As the next step after confirmation, these genes could be transferred to economically important plants to gain boron tolerance. Additionally, possibly Puccinellia specific miRNAs were also identified and these may be important for boron tolerance/hyperaccumulation mechanisms. Not only transcriptional level but also post-transcriptional changes occur in the Puccinellia transcriptome and they are shown in this study. These results provide new information and insights into the underlying boron stress responsive mechanism in shoots and may be applicable for agronomy to develop boron tolerant crop plants. Additionally, using Puccinellia, itself for phytoremediation and/or phytoextraction will provide a new and economical way to remove excess boron from contaminated soil. Therefore this will be useful for soil depollution and for simple boron purification.

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APPENDIX A

DIFFRENTIALLY EXPRESSED TRANSCRIPTS

Transcript Name	Control FPKM	Stress FPKM	Difference between control vs stress
TRINITY_DN174261_c0_g1_i1:0-179	406.0	41806.5	41400.5
TRINITY_DN213899_c1_g5_i1:0-169	365.9	20927.0	20561.1
TRINITY_DN121038_c0_g1_i1:0-299	247.7	18893.6	18645.9
TRINITY_DN174261_c0_g2_i1:0-282	125.0	9078.8	8953.8
TRINITY_DN240298_c0_g1_i1:0-299	96.5	7808.2	7711.7
TRINITY_DN185920_c1_g3_i1:0-163	0.0	3914.9	3914.9
TRINITY_DN111068_c0_g2_i1:14-281	20.0	2281.2	2261.2
TRINITY_DN255535_c0_g1_i1:0-344	34.4	2149.3	2114.8
TRINITY_DN185920_c1_g1_i1:0-270	0.0	1315.7	1315.7
TRINITY_DN237087_c0_g1_i1:45-264	0.0	1102.6	1102.6
TRINITY_DN158617_c0_g3_i1:51-230	0.0	1056.9	1056.9
TRINITY_DN158617_c0_g3_i2:0-344	0.0	1004.4	1004.4
TRINITY_DN169672_c0_g2_i1:37-261	51.8	1028.9	977.1
TRINITY_DN111068_c0_g1_i1:13-283	25.4	988.6	963.2
TRINITY_DN99923_c0_g1_i1:0-171	0.0	845.9	845.9
TRINITY_DN185920_c2_g1_i1:0-1986	0.0	613.7	613.7
TRINITY_DN210950_c1_g3_i1:0-244	35.0	567.0	532.0
TRINITY_DN23532_c0_g1_i1:0-180	0.0	503.9	503.9
TRINITY_DN220021_c1_g4_i1:0-584	104.7	531.0	426.3
TRINITY_DN192927_c1_g1_i1:132-532	82.5	412.1	329.6
TRINITY_DN131860_c0_g2_i1:0-222	0.0	316.0	316.0
TRINITY_DN220021_c1_g2_i1:0-299	28.0	338.5	310.6
TRINITY_DN214561_c1_g7_i1:0-308	32.9	337.8	304.9
TRINITY_DN220021_c1_g4_i2:0-391	33.5	332.8	299.3
TRINITY_DN187809_c1_g2_i1:0-358	19.9	313.4	293.5
TRINITY_DN305698_c0_g1_i1:0-294	0.0	277.0	277.0
TRINITY_DN214561_c1_g18_i1:0-365	27.7	281.3	253.6
TRINITY_DN199272_c0_g3_i1:2-246	0.0	169.0	169.0
TRINITY_DN210741_c3_g13_i1:14-345	19.2	173.4	154.1
TRINITY_DN150574_c0_g1_i1:0-201	0.0	130.1	130.1
TRINITY_DN215985_c1_g3_i1:4-592	23.5	149.8	126.4
TRINITY_DN268357_c0_g1_i1:0-309	0.0	125.5	125.5
TRINITY_DN211139_c12_g1_i2:4-214	0.0	97.7	97.7
TRINITY_DN218921_c2_g11_i1:0-275	0.0	94.5	94.5
TRINITY_DN233649_c0_g1_i1:0-245	0.0	94.4	94.4
TRINITY_DN223632_c5_g9_i1:0-204	0.0	87.0	87.0
TRINITY_DN67858_c0_g1_i1:0-219	0.0	85.6	85.6
TRINITY_DN295912_c0_g1_i1:0-332	0.0	83.3	83.3
TRINITY_DN28983_c0_g1_i1:38-250	0.0	80.8	80.8
TRINITY_DN224067_c1_g1_i1:0-1399	15.8	95.7	79.9
TRINITY_DN206840_c2_g1_i1:32-257	0.0	78.0	78.0
TRINITY_DN194382_c2_g1_i2:0-283	0.0	74.6	74.6
TRINITY_DN156191_c0_g1_i1:0-371	0.0	66.9	66.9

TRINITY_DN211364_c5_g2_i1:0-1033	2.1	68.8	66.7
TRINITY_DN271392_c0_g1_i1:0-479	0.0	66.1	66.1
TRINITY_DN199272_c0_g1_i1:0-245	0.0	65.6	65.6
TRINITY_DN113560_c0_g1_i1:0-238	0.0	63.1	63.1
TRINITY_DN221642_c3_g12_i1:2-232	0.0	57.7	57.7
TRINITY_DN214853_c4_g1_i1:0-762	0.0	57.5	57.5
TRINITY_DN208871_c4_g1_i1:0-352	0.0	55.3	55.3
TRINITY_DN212893_c1_g1_i1:0-261	0.0	53.7	53.7
TRINITY_DN302772_c0_g1_i1:0-218	0.0	52.9	52.9
TRINITY_DN194854_c0_g1_i1:0-255	0.0	52.0	52.0
TRINITY_DN48466_c0_g1_i1:30-384	0.0	51.6	51.6
TRINITY_DN195572_c0_g1_i2:55-342	0.0	49.5	49.5
TRINITY_DN125775_c0_g1_i1:36-294	0.0	48.0	48.0
TRINITY_DN202627_c0_g7_i1:92-353	0.0	45.8	45.8
TRINITY_DN214247_c1_g14_i1:119-359	0.0	44.8	44.8
TRINITY_DN225500_c2_g9_i1:0-236	0.0	40.2	40.2
TRINITY_DN272586_c0_g1_i1:1-261	0.0	39.9	39.9
TRINITY_DN179460_c0_g1_i1:0-227	0.0	39.5	39.5
TRINITY_DN264216_c0_g1_i1:1-257	0.0	38.0	38.0
TRINITY_DN221251_c1_g1_i1:1-258	0.0	34.7	34.7
TRINITY_DN219297_c2_g6_i3:1-241	0.0	34.1	34.1
TRINITY_DN156191_c0_g2_i1:0-342	0.0	32.7	32.7
TRINITY_DN226575_c2_g2_i1:2-256	0.0	32.1	32.1
TRINITY_DN8209_c0_g1_i1:0-419	0.0	31.5	31.5
TRINITY_DN203114_c0_g3_i1:0-256	0.0	30.3	30.3
TRINITY_DN210823_c0_g3_i1:4-269	0.0	30.1	30.1
TRINITY_DN202443_c4_g5_i3:0-460	0.0	29.4	29.4
TRINITY_DN114402_c0_g1_i1:9-274	0.0	29.4	29.4
TRINITY_DN241186_c0_g1_i1:0-1743	2.7	31.9	29.2
TRINITY_DN218938_c0_g2_i1:15-299	0.0	28.3	28.3
TRINITY_DN198354_c0_g2_i1:0-272	0.0	27.2	27.2
TRINITY_DN66009_c0_g1_i1:0-263	0.0	27.0	27.0
TRINITY_DN202443_c4_g4_i2:0-314	0.0	25.9	25.9
TRINITY_DN202443_c4_g4_i3:0-340	0.0	25.5	25.5
TRINITY_DN216242_c2_g1_i3:1-273	0.0	25.3	25.3
TRINITY_DN219995_c1_g16_i1:0-256	0.0	25.1	25.1
TRINITY_DN226626_c0_g8_i1:3-253	0.0	24.8	24.8
TRINITY_DN211426_c2_g4_i3:0-258	0.0	24.3	24.3
TRINITY_DN202443_c4_g1_i1:0-255	0.0	24.2	24.2
TRINITY_DN226234_c5_g4_i1:1-277	0.0	23.9	23.9
TRINITY_DN306660_c0_g1_i1:0-326	0.0	22.6	22.6
TRINITY_DN208926_c0_g1_i1:1-311	0.0	22.3	22.3
TRINITY_DN216505_c0_g1_i2:0-313	0.0	21.9	21.9
TRINITY_DN226592_c4_g15_i1:8-257	0.0	21.5	21.5
TRINITY_DN226529_c6_g13_i2:0-665	3.5	24.2	20.6
TRINITY_DN295721_c0_g1_i1:0-414	0.0	20.4	20.4
TRINITY_DN140573_c0_g2_i1:0-250	0.0	20.3	20.3
TRINITY_DN179003_c1_g2_i1:7-283	0.0	20.1	20.1
TRINITY_DN294520_c0_g1_i1:0-828	0.7	20.8	20.0
TRINITY_DN278982_c0_g1_i1:1-268	0.0	19.7	19.7
TRINITY_DN284184_c0_g1_i1:0-703	0.0	19.5	19.5
TRINITY_DN222292_c3_g2_i1:0-281	0.0	18.7	18.7
TRINITY_DN1712_c0_g1_i1:0-285	0.0	18.6	18.6

TRINITY_DN143038_c0_g1_i1:0-301	0.0	18.6	18.6
TRINITY_DN225050_c0_g11_i1:0-252	0.0	18.3	18.3
TRINITY_DN213110_c2_g2_i4:22-323	0.0	17.0	17.0
TRINITY_DN186267_c2_g1_i1:18-638	0.0	16.9	16.9

APPENDIX B

SOURCE BASED MiRNAs

mirnaName	mirnaSourceAccession	Target_Accession	mirna Source Description	Target Description	Difference between control vs stress (mirna source)
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN107030_c0_g1_i1	---NA---	A8MQN4_ARATHCalcineurin-like metallo-phosphoesterase superfamily OS=Arabidopsis thaliana GN=At1g53710 PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN107030_c0_g2_i1	---NA---	A8MQN4_ARATHCalcineurin-like metallo-phosphoesterase superfamily OS=Arabidopsis thaliana GN=At1g53710 PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN123195_c0_g1_i1	---NA---	BRADI C3SA74 F-box domain containing OS=Brachypodium distachyon PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN123195_c0_g2_i1	---NA---	BRADI C3SA74 F-box domain containing OS=Brachypodium distachyon PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN142752_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN142872_c0_g2_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN142872_c0_g3_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN147174_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN170137_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN174605_c1_g1_i1	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN181084_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN181084_c0_g2_i1	---NA---	A0A0H3VDJ0_ORYSALipid transfer -2 OS=Oryza sativa GN=LTP2 PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN181084_c0_g2_i2	---NA---	A0A0H3VDJ0_ORYSALipid transfer -2 OS=Oryza sativa GN=LTP2 PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN184280_c0_g1_i1	---NA---	AEGTA M8CAP2 Uncharacterized protein OS=Aegilops tauschii GN=F775_00171 PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN186427_c1_g4_i1	---NA---	---NA---	27.0

pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN191555_c0_g1_i2	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN197754_c1_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN197754_c1_g2_i2	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN197754_c1_g3_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN199757_c0_g1_i1	---NA---	TOP1B_ARATHDNA topoisomerase 1 beta OS=Arabidopsis thaliana GN=TOP1B PE=1 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN199757_c0_g1_i3	---NA---	TOP1B_ARATHDNA topoisomerase 1 beta OS=Arabidopsis thaliana GN=TOP1B PE=1 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN203133_c0_g1_i2	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN204548_c1_g1_i1	---NA---	SC31B_ARATH transport SEC31 homolog B OS=Arabidopsis thaliana GN=SEC31B PE=1 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN207459_c1_g1_i1	---NA---	A8MQN4_ARATHCalcineurin-like metallo-phosphoesterase superfamily OS=Arabidopsis thaliana GN=At1g53710 PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN207825_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN209123_c1_g2_i1	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN209123_c1_g2_i2	---NA---	HORVD F2DDD3 OS=Hordeum vulgare distichum PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN209123_c1_g2_i3	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN210440_c1_g1_i1	---NA---	Q501A1_ARATHAt2g18860 OS=Arabidopsis thaliana GN=At2g18860 PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN210440_c1_g1_i2	---NA---	Q501A1_ARATHAt2g18860 OS=Arabidopsis thaliana GN=At2g18860 PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN211704_c2_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN211704_c2_g2_i2	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN211704_c2_g6_i3	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN212567_c2_g9_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN212953_c1_g3_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN213005_c0_g1_i1	---NA---	P2C19_ARATH phosphatase 2C and cyclic nucleotide-binding kinase domain-containing OS=Arabidopsis thaliana GN=At2g20050 At2g20040 PE=2 SV=2	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN214110_c0_g1_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN215296_c4_g1_i1	---NA---	---NA---	27.0

pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN215296_c4_g1_i2	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN215296_c4_g1_i3	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN216077_c0_g1_i1	---NA---	SCKL1_ARATHFructokinase-like chloroplastic OS=Arabidopsis thaliana GN=FLN1 PE=1 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN217241_c1_g2_i1	---NA---	WHEAT W5F8Y7 Uncharacterized protein OS=Triticum aestivum PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN217316_c1_g4_i1	---NA---	NDF5_ARATH NDH-DEPENDENT CYCLIC ELECTRON FLOW 5 OS=Arabidopsis thaliana GN=NDF5 PE=2 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN218284_c1_g3_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN218455_c1_g1_i1	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN218649_c3_g2_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN221088_c5_g1_i1	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN221088_c5_g1_i3	---NA---	OS=Oryza sativa GN= PE=4 SV=1	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN221527_c1_g10_i1	---NA---	---NA---	27.0
pdi-mir-42	TRINITY_DN66009_c0_g1_i1	TRINITY_DN222683_c2_g10_i1	---NA---	---NA---	27.0

Only 50 of them are presented, for remaining visit: “http://plantmolgen.iyte.edu.tr/”

APPENDIX C

TARGET BASED MiRNAs

Mirna Name	mirnaSource Accession	Target Accession	mirna Source Description	Target Description	Difference between control vs stress (target)
pdi-mir-6340	TRINITY_DN162939_c3_g1_i1	TRINITY_DN174261_c0_g1_i1	---NA---	---NA---	41400.5
pdi-mir-6340	TRINITY_DN162939_c3_g1_i1	TRINITY_DN174261_c0_g2_i1	---NA---	---NA---	8953.8
pdi-mir-3853	TRINITY_DN222920_c2_g7_i1	TRINITY_DN111068_c0_g2_i1	---NA---	---NA---	2261.2
pdi-mir-3853	TRINITY_DN222920_c2_g7_i1	TRINITY_DN111068_c0_g1_i1	---NA---	---NA---	963.2
pdi-mir-3399	TRINITY_DN209063_c1_g1_i4	TRINITY_DN99923_c0_g1_i1	GSTFB_ARATHGlutathione S-transferase F11 OS=Arabidopsis thaliana GN=GSTF11 PE=2 SV=1	H32_ARATHHistone OS=Arabidopsis thaliana GN=HTR2 PE=1 SV=2	845.9
pdi-mir-11569	TRINITY_DN200167_c0_g1_i1	TRINITY_DN185920_c2_g1_i1	Q8GY46_ARATHAt5g13560 OS=Arabidopsis thaliana GN=At5g13560 PE=1 SV=1	---NA---	613.7
pdi-mir-17707	TRINITY_DN32374_c0_g2_i1	TRINITY_DN185920_c2_g1_i1	---NA---	---NA---	613.7
pdi-mir-23056	TRINITY_DN137720_c1_g1_i1	TRINITY_DN185920_c2_g1_i1	POB1_ARATHBTB POZ domain-containing POB1 OS=Arabidopsis thaliana GN=POB1 PE=2 SV=2	---NA---	613.7
pdi-mir-3330	TRINITY_DN192927_c2_g1_i1	TRINITY_DN192927_c1_g1_i1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	329.6
pdi-mir-3404	TRINITY_DN192927_c2_g1_i1	TRINITY_DN192927_c1_g1_i1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	329.6
pdi-mir-12244	TRINITY_DN212040_c3_g1_i2	TRINITY_DN192927_c1_g1_i1	---NA---	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	329.6

pdi-mir-14028	TRINITY_DN192927_c2_g1_i1	TRINITY_DN192927_c1_g1_i1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	329.6
pdi-mir-3509	TRINITY_DN225818_c3_g1_i1	TRINITY_DN131860_c0_g2_i1	---NA---	RD22_ARATHBURP domain RD22 OS=Arabidopsis thaliana GN=RD22 PE=2 SV=1	316.0
pdi-mir-14122	TRINITY_DN219270_c1_g1_i1	TRINITY_DN131860_c0_g2_i1	RD22_ARATHBURP domain RD22 OS=Arabidopsis thaliana GN=RD22 PE=2 SV=1	RD22_ARATHBURP domain RD22 OS=Arabidopsis thaliana GN=RD22 PE=2 SV=1	316.0
pdi-mir-4191	TRINITY_DN218389_c0_g2_i1	TRINITY_DN214561_c1_g7_i1	SPT51_ARATH transcription elongation factor SPT5 homolog 1 OS=Arabidopsis thaliana GN=At4g08350 PE=1 SV=2	---NA---	304.9
pdi-mir-4787	TRINITY_DN210694_c0_g1_i1	TRINITY_DN214561_c1_g7_i1	---NA---	---NA---	304.9
pdi-mir-486	TRINITY_DN155995_c0_g1_i1	TRINITY_DN211139_c12_g1_i2	---NA---	---NA---	97.7
pdi-mir-6340	TRINITY_DN162939_c3_g1_i1	TRINITY_DN211139_c12_g1_i2	---NA---	---NA---	97.7
pdi-mir-3287	TRINITY_DN192779_c0_g1_i1	TRINITY_DN224067_c1_g1_i1	TRIUA M7ZLF2 Serine carboxypeptidase-like 6 OS=Triticum urartu GN=TRIUR3_09962 PE=3 SV=1	FRO7_ARATHFerric reduction oxidase chloroplastic OS=Arabidopsis thaliana GN=FRO7 PE=2 SV=1	79.9
pdi-mir-11262	TRINITY_DN161996_c0_g1_i1	TRINITY_DN224067_c1_g1_i1	CIPK1_ARATHCBL-interacting serine threonine-kinase 1 OS=Arabidopsis thaliana GN=CIPK1 PE=1 SV=2	FRO7_ARATHFerric reduction oxidase chloroplastic OS=Arabidopsis thaliana GN=FRO7 PE=2 SV=1	79.9
pdi-mir-2980	TRINITY_DN210056_c3_g3_i1	TRINITY_DN206840_c2_g1_i1	---NA---	BRADI I1GZH5 Uncharacterized protein OS=Brachypodium distachyon GN=BRADI_1g45000 PE=4 SV=1	78.0
pdi-mir-763	TRINITY_DN203521_c0_g4_i1	TRINITY_DN156191_c0_g1_i1	---NA---	RICCO B9SDT6 Inter-alpha-trypsin inhibitor heavy chain OS=Ricinus communis GN=RCOM_1711500 PE=4 SV=1	66.9
pdi-mir-21233	TRINITY_DN212296_c0_g5_i1	TRINITY_DN156191_c0_g1_i1	PP295_ARATHPentatricopeptide repeat-containing At3g62890 OS=Arabidopsis thaliana GN=PCMP-H82 PE=2 SV=1	RICCO B9SDT6 Inter-alpha-trypsin inhibitor heavy chain OS=Ricinus communis GN=RCOM_1711500 PE=4 SV=1	66.9
pdi-mir-10937	TRINITY_DN224646_c3_g6_i6	TRINITY_DN211364_c5_g2_i1	ANACO A0A199V1B5 MACPF domain-containing OS=Ananas comosus GN=ACMD2_22687 PE=4 SV=1	---NA---	66.7
pdi-mir-6082	TRINITY_DN147864_c0_g2_i1	TRINITY_DN271392_c0_g1_i1	---NA---	H32_ARATHHistone OS=Arabidopsis thaliana GN=HTR2 PE=1 SV=2	66.1

pdi-mir-12783	TRINITY_DN221500_c4_g1_i4	TRINITY_DN221642_c3_g12_i1	---NA---	XTH22_ARATHXyloglucan endotransglucosylase hydrolase 22 OS=Arabidopsis thaliana GN=XTH22 PE=1 SV=1	57.7
pdi-mir-15815	TRINITY_DN303363_c0_g1_i1	TRINITY_DN221642_c3_g12_i1	---NA---	XTH22_ARATHXyloglucan endotransglucosylase hydrolase 22 OS=Arabidopsis thaliana GN=XTH22 PE=1 SV=1	57.7
pdi-mir-19983	TRINITY_DN222981_c2_g1_i3	TRINITY_DN194854_c0_g1_i1	A0A0U2QI38_ORYSANBS-LRR-like resistance OS=Oryza sativa GN=Os07g04900 PE=4 SV=1	AEGTA R7W1U0 Boron transporter 2 OS=Aegilops tauschii GN=F775_15590 PE=4 SV=1	52.0
pdi-mir-13425	TRINITY_DN219590_c2_g1_i2	TRINITY_DN195572_c0_g1_i2	Q8VZ71_ARATHAT2G31560 OS=Arabidopsis thaliana GN=At2g31560 PE=2 SV=1	---NA---	49.5
pdi-mir-19873	TRINITY_DN213036_c3_g4_i1	TRINITY_DN214247_c1_g14_i1	---NA---	WHEAT W5G868 Uncharacterized protein OS=Triticum aestivum PE=4 SV=1	44.8
pdi-mir-13215	TRINITY_DN146213_c0_g1_i1	TRINITY_DN272586_c0_g1_i1	---NA---	ACT3_ARATHActin-3 OS=Arabidopsis thaliana GN=ACT3 PE=1 SV=1	39.9
pdi-mir-13370	TRINITY_DN208725_c0_g1_i3	TRINITY_DN272586_c0_g1_i1	---NA---	ACT3_ARATHActin-3 OS=Arabidopsis thaliana GN=ACT3 PE=1 SV=1	39.9
pdi-mir-17756	TRINITY_DN214115_c1_g1_i1	TRINITY_DN264216_c0_g1_i1	---NA---	COX3_ARATHCytochrome c oxidase subunit 3 OS=Arabidopsis thaliana GN=COX3 PE=2 SV=2	38.0
pdi-mir-6085	TRINITY_DN210469_c1_g1_i2	TRINITY_DN221251_c1_g1_i1	OS=Oryza sativa GN= PE=4 SV=1	---NA---	34.7
pdi-mir-23344	TRINITY_DN221204_c3_g4_i2	TRINITY_DN221251_c1_g1_i1	Q0WPD9_ARATHPutative uncharacterized protein At3g11620 OS=Arabidopsis thaliana GN=At3g11620 PE=2 SV=1	---NA---	34.7
pdi-mir-19679	TRINITY_DN223478_c0_g2_i2	TRINITY_DN219297_c2_g6_i3	---NA---	HORVD M0XHD4 Uncharacterized protein OS=Hordeum vulgare var. distichum PE=4 SV=1	34.1
pdi-mir-23299	TRINITY_DN223478_c0_g2_i2	TRINITY_DN219297_c2_g6_i3	---NA---	HORVD M0XHD4 Uncharacterized protein OS=Hordeum vulgare var. distichum PE=4 SV=1	34.1
pdi-mir-763	TRINITY_DN203521_c0_g4_i1	TRINITY_DN156191_c0_g2_i1	---NA---	RICCO B9SDT6 Inter-alpha-trypsin inhibitor heavy chain OS=Ricinus communis GN=RCOM_1711500 PE=4 SV=1	32.7
pdi-mir-21233	TRINITY_DN212296_c0_g5_i1	TRINITY_DN156191_c0_g2_i1	PP295_ARATHPentatricopeptide repeat-containing At3g62890 OS=Arabidopsis thaliana GN=PCMP-H82 PE=2 SV=1	RICCO B9SDT6 Inter-alpha-trypsin inhibitor heavy chain OS=Ricinus	32.7

				communis GN=RCOM_1711500 PE=4 SV=1	
pdi-mir-12885	TRINITY_DN220999_c0_g1_i3	TRINITY_DN210823_c0_g3_i1	OS=Oryza sativa GN= PE=4 SV=1	Q949A7_ORYSA aquaporin (Fragment) OS=Oryza sativa GN=C1275ERIPDM PE=3 SV=2	30.1
pdi-mir-2389	TRINITY_DN215646_c1_g8_i1	TRINITY_DN114402_c0_g1_i1	---NA---	FRS5_ARATH FAR1-RELATED SEQUENCE 5 OS=Arabidopsis thaliana GN=FRS5 PE=2 SV=1	29.4
pdi-mir-5548	TRINITY_DN158662_c0_g1_i1	TRINITY_DN241186_c0_g1_i1	---NA---	EF1A4_ARATHElongation factor 1-alpha 4 OS=Arabidopsis thaliana GN=A4 PE=1 SV=2	29.2
pdi-mir-12458	TRINITY_DN212712_c2_g1_i1	TRINITY_DN241186_c0_g1_i1	SCP18_ARATHSerine carboxypeptidase-like 18 OS=Arabidopsis thaliana GN=SCPL18 PE=2 SV=2	EF1A4_ARATHElongation factor 1-alpha 4 OS=Arabidopsis thaliana GN=A4 PE=1 SV=2	29.2
pdi-mir-3031	TRINITY_DN215504_c0_g2_i1	TRINITY_DN218938_c0_g2_i1	TAF2_ARATHTranscription initiation factor TFIID subunit 2 OS=Arabidopsis thaliana GN=TAF2 PE=2 SV=1	HORVD F2EES7 OS=Hordeum vulgare distichum PE=2 SV=1	28.3
pdi-mir-14247	TRINITY_DN222183_c0_g1_i2	TRINITY_DN218938_c0_g2_i1	CSLG2_ARATHCellulose synthase G2 OS=Arabidopsis thaliana GN=CSLG2 PE=2 SV=1	HORVD F2EES7 OS=Hordeum vulgare distichum PE=2 SV=1	28.3
pdi-mir-10400	TRINITY_DN203656_c1_g1_i5	TRINITY_DN216242_c2_g1_i3	---NA---	---NA---	25.3
pdi-mir-15566	TRINITY_DN203656_c1_g1_i5	TRINITY_DN216242_c2_g1_i3	---NA---	---NA---	25.3
pdi-mir-2170	TRINITY_DN210514_c1_g3_i1	TRINITY_DN219995_c1_g16_i1	---NA---	Q9LK47_ARATHAT3g23700 MYM9_3 OS=Arabidopsis thaliana GN=At3g23700 PE=2 SV=1	25.1
pdi-mir-11990	TRINITY_DN224011_c1_g2_i3	TRINITY_DN226626_c0_g8_i1	HORVD F2DJQ1 OS=Hordeum vulgare distichum PE=2 SV=1	A0A0K0WRF9_ORYSANBS-LRR type R OS=Oryza sativa GN=Pi50_NBS4_3 PE=4 SV=1	24.8
pdi-mir-19034	TRINITY_DN211426_c2_g4_i1	TRINITY_DN211426_c2_g4_i3	Q8W264_ORYSAEndo-1,3-beta-glucanase OS=Oryza sativa PE=2 SV=1	---NA---	24.3

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APPENDIX D

ANNOTATION OF DIFFERENTIALLY EXPRESSED TRANSCRIPTS

TranscriptName	Sequence Description	FPKM (Difference)
TRINITY_DN213899_c1_g5_i1:0-169	F4I600_ARATHV-ATPase-related OS=Arabidopsis thaliana GN=At1g16820 PE=4 SV=1	20561.12
TRINITY_DN99923_c0_g1_i1:0-171	H32_ARATHHistone OS=Arabidopsis thaliana GN=HTR2 PE=1 SV=2	845.90
TRINITY_DN220021_c1_g4_i1:0-584	RRAA2_ARATH 4-hydroxy-4-methyl-2-oxoglutarate aldolase 2 OS=Arabidopsis thaliana GN=At5g16450 PE=1 SV=1	426.28
TRINITY_DN192927_c1_g1_i1:132-532	PSBR_ARATHPhotosystem II 10 kDa chloroplastic OS=Arabidopsis thaliana GN=PSBR PE=1 SV=1	329.61
TRINITY_DN131860_c0_g2_i1:0-222	RD22_ARATHBURP domain RD22 OS=Arabidopsis thaliana GN=RD22 PE=2 SV=1	315.95
TRINITY_DN220021_c1_g4_i2:0-391	RRAA3_ARATH 4-hydroxy-4-methyl-2-oxoglutarate aldolase 3 OS=Arabidopsis thaliana GN=At5g56260 PE=1 SV=1	299.33
TRINITY_DN214561_c1_g18_i1:0-365	RRAA3_ARATH 4-hydroxy-4-methyl-2-oxoglutarate aldolase 3 OS=Arabidopsis thaliana GN=At5g56260 PE=1 SV=1	253.61
TRINITY_DN199272_c0_g3_i1:2-246	H2B10_ARATHHistone OS=Arabidopsis thaliana GN=At5g22880 PE=1 SV=3	168.98
TRINITY_DN150574_c0_g1_i1:0-201	H2B1_ARATHHistone OS=Arabidopsis thaliana GN=At1g07790 PE=1 SV=3	130.09
TRINITY_DN215985_c1_g3_i1:4-592	A0A160DR54_ORYSADirigent (Fragment) OS=Oryza sativa PE=3 SV=1	126.38
TRINITY_DN67858_c0_g1_i1:0-219	RL40B_ARATHUbiquitin-60S ribosomal L40-2 OS=Arabidopsis thaliana GN=RPL40B PE=2 SV=2	85.64
TRINITY_DN28983_c0_g1_i1:38-250	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	80.83
TRINITY_DN224067_c1_g1_i1:0-1399	FRO7_ARATHFerric reduction oxidase chloroplastic OS=Arabidopsis thaliana GN=FRO7 PE=2 SV=1	79.87
TRINITY_DN271392_c0_g1_i1:0-479	H32_ARATHHistone OS=Arabidopsis thaliana GN=HTR2 PE=1 SV=2	66.13
TRINITY_DN199272_c0_g1_i1:0-245	H2B3_ARATHHistone OS=Arabidopsis thaliana GN=At2g28720 PE=1 SV=3	65.63
TRINITY_DN113560_c0_g1_i1:0-238	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	63.07
TRINITY_DN221642_c3_g12_i1:2-232	XTH22_ARATHXyloglucan endotransglucosylase hydrolase 22 OS=Arabidopsis thaliana GN=XTH22 PE=1 SV=1	57.72
TRINITY_DN48466_c0_g1_i1:30-384	H32_ARATHHistone OS=Arabidopsis thaliana GN=HTR2 PE=1 SV=2	51.59
TRINITY_DN202627_c0_g7_i1:92-353	Q01KB9_ORYSAAutophagy-related OS=Oryza sativa GN= PE=3 SV=1	45.84

TRINITY_DN272586_c0_g1_i1:1-261	ACT3_ARATHActin-3 OS=Arabidopsis thaliana GN=ACT3 PE=1 SV=1	39.89
TRINITY_DN179460_c0_g1_i1:0-227	SYHM_ARATHHistidine--tRNA chloroplastic mitochondrial OS=Arabidopsis thaliana GN=At3g46100 PE=2 SV=1	39.49
TRINITY_DN264216_c0_g1_i1:1-257	COX3_ARATHCytochrome c oxidase subunit 3 OS=Arabidopsis thaliana GN=COX3 PE=2 SV=2	38.02
TRINITY_DN226575_c2_g2_i1:2-256	OS=Oryza sativa GN= PE=4 SV=1	32.09
TRINITY_DN8209_c0_g1_i1:0-419	H4_ARATHHistone H4 OS=Arabidopsis thaliana GN=At1g07660 PE=1 SV=2	31.53
TRINITY_DN203114_c0_g3_i1:0-256	OS=Oryza sativa GN= PE=4 SV=1	30.27
TRINITY_DN210823_c0_g3_i1:4-269	Q949A7_ORYSA aquaporin (Fragment) OS=Oryza sativa GN=C1275ERIPDM PE=3 SV=2	30.12
TRINITY_DN202443_c4_g5_i3:0-460	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	29.44
TRINITY_DN114402_c0_g1_i1:9-274	FRS5_ARATH FAR1-RELATED SEQUENCE 5 OS=Arabidopsis thaliana GN=FRS5 PE=2 SV=1	29.40
TRINITY_DN241186_c0_g1_i1:0-1743	OS=Oryza sativa GN= PE=4 SV=1	29.18
TRINITY_DN202443_c4_g4_i2:0-314	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	25.88
TRINITY_DN202443_c4_g4_i3:0-340	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	25.53
TRINITY_DN219995_c1_g16_i1:0-256	Q9LK47_ARATHAT3g23700 MYM9_3 OS=Arabidopsis thaliana GN=At3g23700 PE=2 SV=1	25.08
TRINITY_DN226626_c0_g8_i1:3-253	A0A0K0WRF9_ORYSANBS-LRR type R OS=Oryza sativa GN=Pi50_NBS4_3 PE=4 SV=1	24.76
TRINITY_DN202443_c4_g1_i1:0-255	H2A1_ARATHProbable histone OS=Arabidopsis thaliana GN=At1g51060 PE=1 SV=1	24.25
TRINITY_DN216505_c0_g1_i2:0-313	OS=Oryza sativa GN= PE=4 SV=1	21.92
TRINITY_DN226592_c4_g15_i1:8-257	NEN3_ARATH NEN3 OS=Arabidopsis thaliana GN=NEN3 PE=2 SV=1	21.50
TRINITY_DN295721_c0_g1_i1:0-414	RL341_ARATH60S ribosomal L34-1 OS=Arabidopsis thaliana GN=RPL34A PE=2 SV=1	20.41
TRINITY_DN140573_c0_g2_i1:0-250	F4HS76_ARATHGlycerol kinase OS=Arabidopsis thaliana GN=NHO1 PE=4 SV=1	20.29
TRINITY_DN179003_c1_g2_i1:7-283	RL142_ARATH60S ribosomal L14-2 OS=Arabidopsis thaliana GN=RPL14B PE=1 SV=1	20.12
TRINITY_DN294520_c0_g1_i1:0-828	RL74_ARATH60S ribosomal L7-4 OS=Arabidopsis thaliana GN=RPL7D PE=2 SV=1	20.02
TRINITY_DN278982_c0_g1_i1:1-268	F4IJE1_ARATHPhox domain-containing OS=Arabidopsis thaliana GN=At2g15900 PE=1 SV=1	19.68
TRINITY_DN284184_c0_g1_i1:0-703	COX2_ARATHCytochrome c oxidase subunit 2 OS=Arabidopsis thaliana GN=COX2 PE=1 SV=2	19.48
TRINITY_DN222292_c3_g2_i1:0-281	Y1457_ARATHAcyltransferase chloroplastic OS=Arabidopsis thaliana GN=At1g54570 PE=2 SV=1	18.69
TRINITY_DN1712_c0_g1_i1:0-285	Q9LTW8_ARATHCTP synthase OS=Arabidopsis thaliana GN=emb2742 PE=2 SV=1	18.65
TRINITY_DN225050_c0_g11_i1:0-252	OS=Oryza sativa GN= PE=4 SV=1	18.33
TRINITY_DN213110_c2_g2_i4:22-323	A0A0U2JFK9_ORYSANBS-LRR-like resistance OS=Oryza sativa GN=Os05g40150 PE=4 SV=1	16.98
TRINITY_DN186267_c2_g1_i1:18-638	UBQ3_ARATHPolyubiquitin 3 OS=Arabidopsis thaliana GN=UBQ3 PE=2 SV=1	16.88
TRINITY_DN204305_c3_g6_i2:0-375	ACT11_ARATHActin-11 OS=Arabidopsis thaliana GN=ACT11 PE=1 SV=1	16.84

TRINITY_DN224769_c1_g7_i1:4-287	A8MRY7_ARATHWD-40 PCN OS=Arabidopsis thaliana GN=At4g07410 PE=4 SV=1	15.55

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VITA

Saniye Elvan ÖZTÜRK

Date and Place of Birth: 1989 Menteşe/ MUĞLA

E-mail : elvanbaskurt4848@gmail.com

Education

Ph.D. Molecular Biology and Genetics
20014-2017 Izmir Institute of Technology,
Molecular Biology and Genetics Department, Izmir, Turkey

MSc. Molecular Biology and Genetics
2011-2014 Izmir Institute of Technology,
Molecular Biology and Genetics Department, Izmir, Turkey

BSc. Molecular Biology and Genetics
2007 – 2011 Istanbul University,
Molecular Biology and Genetics Department, Istanbul, Turkey

Teaching and Professional Experience

Research assistant

2011-2017 Izmir Institute of Technology,
Molecular Biology and Genetics Department, Izmir, Turkey
2011-… Aksaray University
Biotechnology and Molecular Biology Department, Aksaray, Turkey

Publications

Ozturk SE, Goktay M, Has C, Babaoglu M, Allmer J, Doganlar S, Frary A. (2017). Boron Hyperaccumulation Mechanisms in Puccinellia distans as Revealed by Transcriptomic Analysis. bioRxiv, 110403.

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