ELUCIDATION OF BORON HYPERACCUMULATION AND TOLERANCE MECHANISMS IN *PUCCINELLIA DISTANS* (Jacq.) PARL. USING PROTEOMICS APPROACH

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
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July 2015
İZMİR

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my supervisors Prof. Dr. Anne FRARY and Prof. Dr. Sami DOĞANLAR for their guidance, patience and unique support and contributions during my M.Sc. period.

I would like to thank all my fellow colleagues whom I have worked with in Doganlar/Frary Plant Molecular Genetics and Breeding Laboratory.

I would like to thank TUBITAK for providing financial support for our project.

I would like to express my sincere gratitude to Prof. Dr. Sebastien Carpentier and Laboratory technician Kusay Arat for putting up with all my requests and hosting me in their laboratories and Facility for Systems Biology Based Mass Spectrometry through my research in Leuven, Belgium.

I would like to thank Canan Has from Izmir Institute of Technology Bioinformatics laboratory for helping me so much, and putting up with all my never-ending questions.

I would like to give special thanks and love to my family, who has never stopped supporting me even in hard times, and also to my dear friends who were always there through joy and sadness.

ABSTRACT

ELUCIDATION OF BORON HYPERACCUMULATION AND TOLERANCE MECHANISMS IN *PUCCINELLIA DISTANS* (Jacq.) PARL. USING PROTEOMICS APPROACH

Boron can cause growth deficiency and yield loss in plants at both low and high concentrations. Turkey holds 72,5% of known world boron reserves, and boron toxicity causes major agricultural problems. In a search to identify resistant species, *Puccinellia distans* (weeping alkaligrass), growing near boron mining sites in Turkey, was found to tolerate toxic boron concentrations. This species has been studied at the level of gene transcription, however, investigation at the protein level has not yet been performed. In this work, the boron stress tolerance mechanism of *P. distans* was investigated with proteomic approaches. For this purpose, plants were divided into control and stress groups with optimal and toxic boron concentrations, respectively. Boron accumulation was observed in leaf tissues at the end of growing stages. Leaf and root tissues were checked for tissue boron concentrations at the end of their growth period with ICP-MS. Both leaves and roots were found to accumulate around 6 g B/kg dry tissue. Total proteins were extracted from leaf tissues of both groups and analyzed in LC-MS instrument to determine the differences between protein profiles. One hundred and nine significantly differentially expressed proteins were identified in this work. Three upregulated proteins were selected as candidate proteins for boron hyperaccumulation tolerance. This work proved successful in identification of some of the proteins responsible for boron hyperaccumulation in *P.distans* and paving the way for future studies to use *P. distans* for phytoremediation in areas with excess boron.

ÖZET

PUCCINELLIA DISTANS (Jacq.) PARL.'DA BORON HİPER-AKÜMÜLASYONU VE TOLERANS MEKANİZMALARININ PROTEOMİKS YAKLAŞIM KULLANARAK ANLAŞILMASI

Bor hem yüksek hem düşük yoğunluklarında bitkilerde büyüme bozukluklarına ve verim kaybına neden olmaktadır. Dünya bor rezervlerinin %72,5'ine sahip olan Türkiye'de bor toksisitesi, önemli bir sorundur ve ülkenin belirli bölgelerindeki birçok tarım bitkisinin verimini azaltmakta ve kullanılabilir tarım alanlarını kısıtlamaktadır. Dayanıklı tür belirlemeye yönelik yapılan araştırmalar sonucunda Türkiye'de bor madenlerinin bulunduğu arazilerde büyüyebilen ve yüksek bor toksisitesine tolerans gösteren *Puccinellia distans* (çorak çimi) tanımlanmıştır. Bu bitki türü ile transkriptomik düzeyde çalışılmış olmasına rağmen daha önce proteomik düzeyde herhangi bir çalışma gerçekleştirilmemiştir. Bu çalışmada, *P. distans* bitkisindeki bor toksisitesine karşı toleransta rol oynayan mekanizmalar proteomik yaklaşımla incelenmiştir. Bu amaca ulaşmak için bitkiler kontrol (optimal bor konsantrasyonlu ortam) ve stres (toksik bor konsantrasyonlu ortam) olarak iki gruba ayrılmış, büyüme evreleri sonunda yaprak dokularında bor biriktirdiği gözlemlenmiştir. Hem yaprakların hem de köklerin yaklaşık 6 gB/kg kuru doku biriktirdiği bulunmuştur. Yaprak dokularından toplam protein ekstraksiyonu gerçekleştirilmiş ve LC-MS yöntemi ile bu grupların protein profillerindeki farklar ile beraber bu farklılıklara neden olan proteinler tanımlanmıştır. Bu çalışmada 189 adet önemli derecede farklı şekilde ifade edilen protein tanımlamıştır. Bunlardan, 3 adedi bor hiper-akümülasyonu için aday proteinler olarak seçilmiştir. Bu yaklaşımla bor hiper-akümülasyonunda görevli olan proteinlerin tanımlanması ve ileride aşırı borlu alanların bitkisel yolla temizlenmezi için *P.distans*'ın kullanımı çalışmalarının önünün açılması amaçlanmaktadır.

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ABBREVIATIONS

2D-PAGE: Two dimensional polyacrylamide gel electrophoresis

ABC: Ammonium bicarbonate

ACN: Acetonitrile

B: Boron

CAN: Acetonitrile

CL: Control leaf

CR: Control Root

BLAST: Basic local alignment sequence tool

BSA: Bovine serum albumin

DDT: Dithiotreitol

EDTA: Ethylenediaminetetraaceticacid

FA: Formic acid

GO: Gene ontology

IAA: Iodoacetamide

ICP-MS: Inductively coupled plasma-mass spectrometry

IPG: Immobilized protein gradient

LC-MS: Liquid chromatography-mass spectrometry

NCBI: National Center for Biotechnology Information

SDS: Sodium dodecyl sulfate

SL: Stress leaf

SR: Stress root

TFA: Trifluoric acid

CHAPTER 1

INTRODUCTION

1.1. Properties of Boron

Boron is the most electronegative element in the 3A group of the periodic table and has one less valance electron in its valance orbital. This feature gives boron properties that make it an important material in the glass, cleaning and ceramic industries (Kot 2009). In addition to its industrial use, boron was found to be an important micronutrient for plant growth and development having roles in: cell wall synthesis and structure, plasma membrane structure, nucleic acid and protein synthesis, carbohydrate metabolism and transport, plant growth regulator metabolism and phenol metabolism (Ahmad et al. 2009, Marschner 1995, Ferrol et al. 1993, Loomis and Durst 1992, Goldbach 1997, Dave 1996, Camacho-Cristobal et al. 2002). Boron was also found to be necessary for human health and trace amounts of boron must be taken daily for healthy metabolic activity (Nielsen 1998).

1.2. Plant Boron Nutrition

1.2.1 Deficiency

Since boron is an important micronutrient, its absence as well as high concentrations have negative effects on organisms. In the absence of boron, leaf growth, root elongation, flower development, fruit and flower formation are impaired and seed yield decreased (Dugger 1983, Dell and Huang 1997, Herrera-Rodríguez et al. 2009). Boron deficiency also results in an imbalance in redox homeostasis throughout the whole plant, increased actin and tubulin concentrations in root cells, and reduced activity of photosynthetic enzymes due to weakening of cell wall structure and photosynthetic enzyme inhibition (Kobayashi et al. 2004, Yu et al. 2003, Han et al. 2008). Boron deficiency in soil can be overcome with easy methods such as fertilizers and soil enrichment but careless use of fertilizers may result in boron toxicity which is also a problem for the growth of plants.

1.2.2 Toxicity

Basic physiological symptoms of boron toxicity in plants were defined as: reduced division of root cells, inhibition of cell wall widening, decrease in chlorophyll content of the leaves and decrease in lignin and suberin content of plant cells (Liu et al. 2000, Nable et al. 1997, Reid 2007). Aside from these symptoms, boron toxicity can also cause chlorosis which may be seen at the tip of old leaves; and in the event of continuous toxicity, chlorosis may turn into necrosis (Tanaka and Fujiwara 2008).

1.3.Distribution of Boron

Boron toxicity is not only caused by over-fertilization of agricultural areas but also due to underground boron reserves. The fact that Turkey possesses 72,5% of world known boron reserves (Figure 1.1) although economically beneficial, causes major problems in agricultural areas.

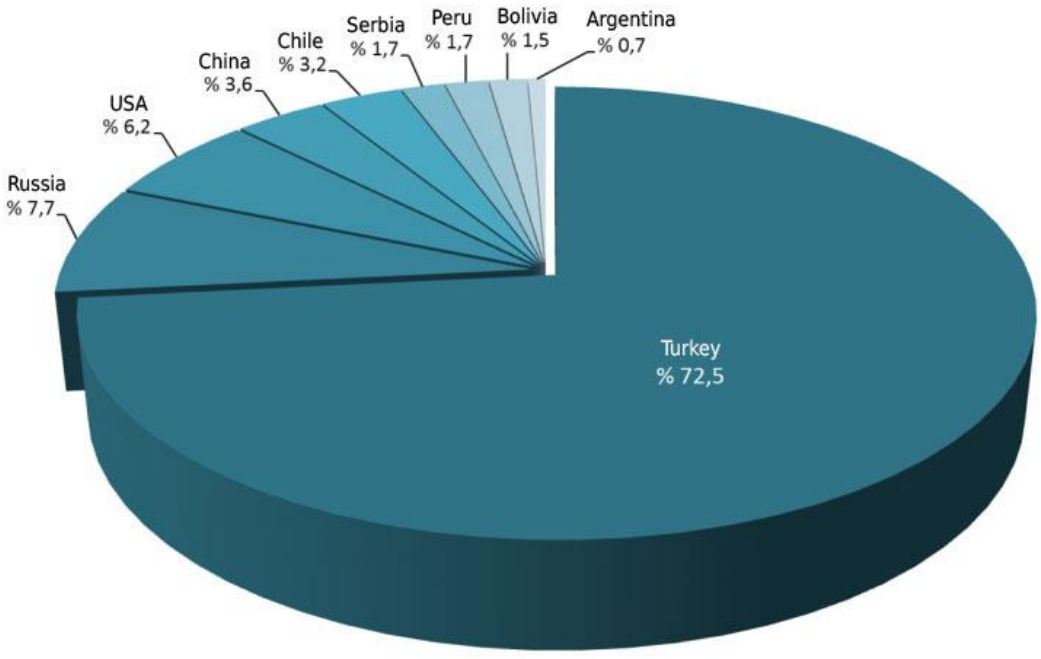

Figure 1.1. Distribution of boron reserves around the World according to National Boron Research Institute. (Source: http://www.boren.gov.tr/en/boron/reserves)

The presence of boron reserves, or their mining activity, both increase the surface concentration of boron. Aside from direct boron concentration in soil, underground water sources and irrigation water around boron mines also carry concentrated amounts of boron to the surface and increase toxic effects on plants (Stiles et al. 2010). Increased boron surface concentration is considered to be a major problem in countries with high boron reserves, impairing agricultural activities. In research performed by Food and Agriculture Organization of the United Nations examining 30 different countries, Iraq, Mexico, Pakistan and Turkey were reported to have reduced agricultural yield due to boron toxicity (Sillanpaa 1990). In more extensive research, boron toxicity was found to negatively affect agricultural production in the Aydın region of Turkey (Koç 2007, Aydın 2012, Soy 2002).

1.4. Solutions to Boron Toxicity

There are three agricultural solutions for boron contaminated soil: leaching, where soil is saturated with water and then drained so that the soluble boron derivatives are also drained (Hoffman 1990); zinc enrichment, where zinc is supplied to the soil as fertilizer to complement the damage caused by boron toxicity; and development of boron tolerant plants. With the leaching method, the amount of water used and how the method is performed must be handled with care since excess treatment with water will cause necessary micronutrients to be carried away. An important limitation of this method is that it cannot be used if boron toxicity is not caused by soluble boron but by insoluble boron (Peryea et al. 1985). As a result of zinc enrichment research, an alternative supplement to zinc was identified as phosphorus to reduce toxic boron symptoms with fertilization (Günes and Alpaslan 2000). Although leaching and zinc enrichment methods are used extensively, they do not provide a permanent solution to boron toxicity and are costly to apply and maintain. So, as a conclusion, development of boron tolerant plants is essential in order to provide a long lasting solution to boron toxicity without disturbing the chemical balance of soil.

1.5.Boron Tolerance Mechanisms

1.5.1 Metabolism

To develop boron tolerant plants, one must first understand the mechanisms of tolerance for such stress. In past studies, it was seen that different plant species develop different stress tolerance mechanisms. In some plants, tolerance of boron is caused by restricted uptake of boron from roots which results in reduced boron accumulation inside the plant, also called a boron resistant plant. It was shown that *Hordeum vulgare* L. and *Triticum aestivum* L. were such plants and can exclude boron from their tissues (Nable 1988, Nable and Paull 1990, Nable et al. 1997). From the work with boron resistant plants which exclude boron from their tissues, it was investigated if tolerance to boron was handled by decreased plasma membrane permeability or because boron was actively pumped outside the cell. In a study where boron resistant Sahara and boron sensitive Schooner barley cultivars were used to understand the basis of this mechanism, it was seen that boron was pumped outside the root cells by an ATP dependent active transport mechanism (Hayes and Reid 2004).

On the other hand, some organisms do not exclude boron from their tissues but accumulate and tolerate them, thus they are called boron accumulator plants. In another study by Stiles et al. (2010) with *Puccinellia distans* and *Gypsophila arrostil*, it was found that boron uptake was unchanged in roots and plants could store boron in shoot and leaf tissues without toxic effects. Since these plants were so good at gathering and storing boron in their leaf tissues, they were called boron accumulator plants. The basis of boron accumulation and tolerance to high concentrations in tissues were not investigated. But it is possible that boron is sequestered inside compartments of the cell or added to the cell wall structure in higher amounts which would reduce its toxic effects to the organism.

1.5.2. Genetics

Genes responsible for boron mobility were first started to be identified through Arabidopsis, a common model organism. Through sequencing of Arabidopsis chromosome 2 (Lin et al. 1999), gene function research picked up the pace. A gene

which is of the bicarbonate transporter superfamily, responsible for boron transport through plasma membrane of pericycle cells in roots was identified (Takano et al. 2001; Frommer and Wirén 2002). In work done by Takano et al. in 2002, this transporter was expressed in yeast cells. It was seen that boron concentration within the cell decreased after transformation of BOR1 gene which led the authors to conclude BOR1 to be an exporter. In research performed by Sutton et al. in 2007, Bot1 an orthologous gene of BOR1 was found to play an important role in boron tolerance mechanisms in high concentrations as well as in low concentrations. In later studies, it was found that both these genes were actually coding transport proteins that can carry boron in and out of the cell (Takano et al. 2008). After Bot1 was identified, more research was done to identify genes that are responsible for boron stress tolerance. In light of these studies, two more boron tolerance genes: TaBOR2 (*T. aestivum*) and HvBOR2 (*H. vulgare*) were found using homology in gene sequence with *Arabidopsis thaliana* and *Oryza sativa* BOR1 genes. Later, probes designed for the gene HvBOR2 were used in Southern blot analysis to find its location on chromosome 4H (Takano 2008, Reid 2007a). As studies continued, it was found that tolerant plants were not only using transport molecules. Certain transcription factors and ribosomal proteins identified in *A. thaliana* were transformed into yeast cells for expression and it was observed that these cells showed increased boron tolerance (Nozawa et al. 2006, Reid 2007b). In previous studies, it was found that boron was able to inhibit *in vitro* pre-mRNA splicing reactions which are thought to be the reason for such tolerance (Reid 2007b). In light of this information, it was postulated that the transcription factors expressed in yeast were able to protect splicing sites and inhibition of these sites was prevented. In another study on *A. thaliana*, a zinc finger protein At1g03770, was found to increase the tolerance of the plant to boron (Kasajima and Fujiwara 2007).

1.6.*Puccinellia distans*

In search of boron tolerant plants, Babaoğlu et al. (2004) identified two plant species around an Eskişehir-Kırka boron mine as boron hyperaccumulators. *G. sphaerocephala,* and *P. distans (Figure 1.2)* were found to be able to accumulate boron up to 3 g and 0.8 g in their leaves, respectively. Given these high levels, both species

became important for research in boron tolerance. Considering that *P. distans* is in the same subfamily with barley and wheat, this plant has the potential for investigation of boron tolerance mechanisms in grass species with agronomical importance as well as for phytoremediation purposes. Also *G. sphaerocephala*, an extreme boron hyperaccumulator, has the potential to be used as a phytoremediating plant where boron taken into the tissues of the plant can later be transported to an area deficient in boron and used as organic fertilizer.

Figure 1.2. *P. distans* plant in Berlin botanical garden. (Source: http://upload.wikimedia .org/wikipedia/commons/4/44/Puccinellia_distans_-_Berlin_Botanical_ Garden_-_IMG_8548.JPG)

In physiological studies performed on *P. distans* and *Gypsophila* species to unlock their potential, it was found that boron was not stored in the root but in the leaves and shoots (Stiles et al. 2010). In later studies, a transcriptomic approach was taken and it was found that four sets of genes, later grouped according to their functions: metabolism, protein synthesis, cell organization and stress tolerance genes, showed higher expression levels than the control group (Padmanabhan et al. 2012). Although there have been molecular studies performed with these plants, neither of them were investigated at the proteomic level and because of this, the effects of post-

translational modifications in the function of proteins to provide boron stress tolerance are still unclear.

1.7.Proteomics

Proteins are large, complex molecules formed by polymerization of amino acids. They function in all metabolic pathways for the continuity of life. Proteins are the end product of the central dogma, for this reason their concentrations may change through time. Depending on the stimuli, synthesis rate of a protein may increase, decrease, be paused or started from a paused position. The stimuli can be external or internal. Through the life cycle of the organism, protein expression will be in constant flux depending on its requirement at that time.

Although mRNA and protein concentrations are related to each other, they are not directly proportional. Considering the complex structure of biological systems there are many factors that affect proportionality. For example, protein post-translational modifications, half-life, and localization are all factors affecting this relationship. Besides the relationship of protein expression to genetic structure, it is well known that protein concentrations change drastically under stress conditions. It is crucial to observe this change in order to determine the basis of tolerance or resistance to the specific stress condition.

Proteomics is a field that studies the changes in protein profile of an organism. It can be either to determine responses to a stress condition or simply to understand the interaction patterns between different proteins. With the versatile tools developed for this area, it is possible to determine protein level changes associated with metabolism, development, stress tolerance/resistance mechanisms and physiological stages.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrel and Patrick 1975) and liquid chromatography-mass spectrometry (LC-MS) (Arpino 1989) are two of a very wide range of techniques used in this field. These techniques are used to elucidate protein profiles in the tissues of an organism. In 2D-PAGE, proteins are separated in the first dimension on an immobilized protein gradient (IPG) strip with mobility depending on their pH. Then, for the second dimension, the strip is rotated and run on an SDS gel with mobility depending on their molecular weight. A better visualization is given in Figure 1.3. While 2D-PAGE is less sensitive to the changes that

occur in the proteome between control and stress treated groups, it provides a general picture as what to expect as a response for that exact stress condition.

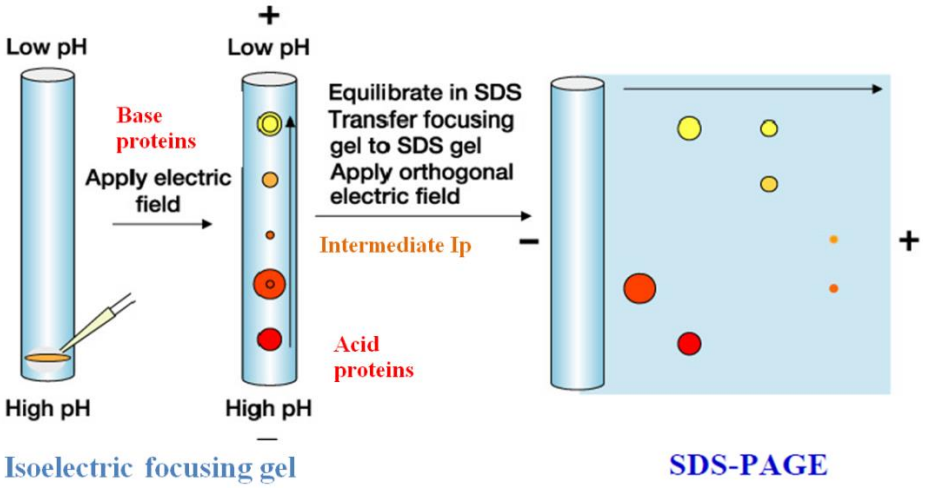

Figure 1.3. Principle of 2D-PAGE.
(Source: http://www.intechopen.com/source/html/38177/media/image4.png)

LC-MS is a more powerful tool to profile the proteome of an organism. As is seen in its name it is performed by coupling liquid chromatography and mass spectrometry (Figure 1.4). First, proteins are separated in LC depending on the desired property with a column. This column may be a hydrophobic or a hydrophilic column which binds hydrophobic and hydrophilic proteins, respectively. Then a solution is passed from the column with a constant rate of change in its property depending on the column. For example, if it is a hydrophobic column, hydrophobicity of the solution is increased with time. Separated proteins are directly given to MS to be analyzed depending on their mass. The power of this tool is that it allows you to make quantitative analysis as well as protein identification depending on the strength of the mass spectrometer.

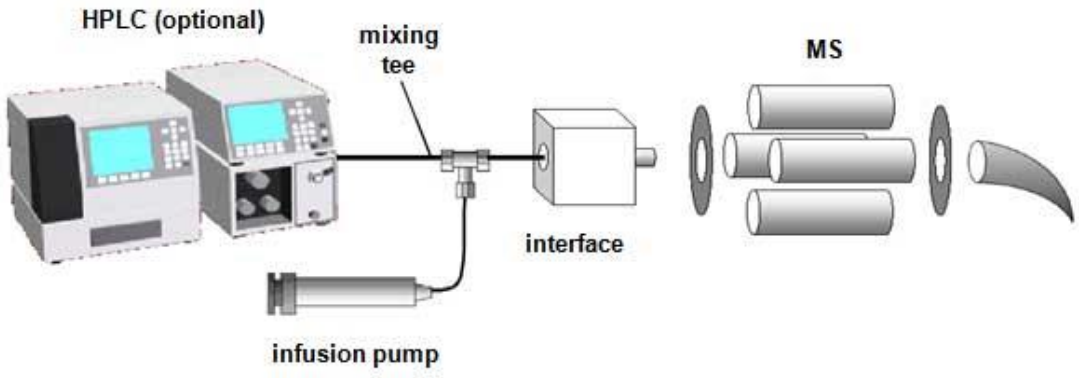

Figure 1.4. Structure of an LC-MS instrument.
(Source: http://www.sepscience.com/images//Articles/HPLCSol/05/Fig-1.jpg)

1.8. Aim of the Experiment

Agricultural precautions taken against boron toxicity are considered temporary and insufficient. For this reason, the best action to take against boron toxicity is the development of boron tolerant plants via plant breeding. In order to be able to reach this future goal, our first objective is to identify and understand boron accumulation and tolerance mechanisms. To do this, we adopted a proteomics approach to observe the changes in protein profiles of plants with and without boron stress application. Due to its extreme tolerance to boron and its ability to accumulate it in its tissues, *P. distans* is considered a suitable plant for this work. Protein profiles were obtained for control and stressed *P. distans* plants using the LC-MS procedure that allowed very sensitive analysis to observe even the slightest changes. This work allowed us to establish basic knowledge for development of plants that can be used for boron phytoremediation on affected soil. Also as a result of this work, we aimed to establish a comprehensive computational proteomics database for annotation of boron tolerance mechanisms for future studies.

CHAPTER 2

MATERIALS AND METHODS

3.1. Plant Growth and Toxic Boron Treatment

P. distans seeds were provided by Prof. Dr. Mehmet Babaoğlu (Selçuk University, Faculty of Agriculture, Department of Field Crops). Seeds were germinated in perlite with Hoagland solution (Hoagland and Amon 1950). Seeds were grown under 25±2°C, 50% humidity and 16/8 light/dark photoperiod for four weeks (Stiles et al. 2010). After four weeks, plants were transferred to half strength Hoagland solution and grown for one week. Plants were separated into two groups with three replicates where 0.5 mg B/L boron concentration was applied to the control group and 500 mg B/L was applied to the stress group (Stiles et al. 2010). Boron concentrations in Hoagland solution were adjusted with boric acid and the solution was changed once every 3 days for 3 weeks. After 3 weeks of treatment, plants were taken out of the half-strength Hoagland solution, both roots and shoots were rinsed of any contamination with distilled water, then the root and shoot tissues were separated from each other to be stored at -80°C.

2.2. Tissue Boron Concentration Determination

Leaf and root tissue samples (1 g) of control and stress groups were ground with liquid nitrogen and placed in a falcon tube. Ground tissue samples were lyophilized for 2 days and analyzed via ICP-MS in Environmental Development Application and Research Center facilities of İzmir Institute of Technology.

2.3. Total Protein Isolation

Protein isolation was performed with the boron treated and untreated *P. distans* leaves. For protein isolation, a phenol isolation method was performed with final protein precipitation using ammonium acetate (Carpentier et al. 2005). The entire extraction procedure was either carried out on ice or at 4°C in centrifuges or -20°C in freezer. Samples were ground with liquid nitrogen in a pre-cooled mortar. After fully

ground, 0,1g powder was transferred to Eppendorf tubes. Immediately, 750 µl extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 30% sucrose, 100 mM KCl, 1% DTT and 1 tablet complete protease inhibitor, pH 8.3) was added onto the samples and mixed. Then 750µl Tris-buffered phenol was added to the mixture and the mixture was vortexed at 4°C for 10 min. Next, samples were centrifuged at 12000 rpm at 4°C for 10 min and phenol phase was transferred to a new tube. Equal volume of extraction buffer was added to the phenol again and centrifuged at 12000 rpm at 4°C for 5 min. Phenol phase was transferred into a new tube and 5 volumes precipitation buffer (0.1 M ammonium acetate in methanol) were added. Samples were incubated overnight in precipitation buffer at -20°C. Samples were then centrifuged at 13000 rpm at 4°C for 60 min and supernatant discarded. Pellet was rinsed with rinsing solution (0.2% DTT in acetone) without resuspending and centrifuged again. On the second rinsing, samples were incubated for 1 h at -20°C then centrifuged. Supernatant was discarded and pellet was dried briefly. Pellet was then suspended in 50-100 µl lysis buffer (8 M urea, 30 mM Tris and prior to use; 5 mM DTT). Samples were vortexed thoroughly and centrifuged at 13000 rpm at 18°C for 30 min. Total protein concentration of the sample within the lysis buffer was measured with Bradford method using a range of BSA of 5-100 µg/µl as standards (Bradford 1976). Proteins were aliquoted to be 20 µg in each tube and stored in -80°C freezer.

2.4. Alkylation of Proteins

Alkylation was performed according to the protocol of the Facility for Systems Biology based Mass Spectrometry (SyBioMa) laboratory of Prof. Dr. Sebastien Carpentier. Stock solutions of 0.2 M DTT, 0.5 M iodoacetamide (IAA), 0.15 M ammonium bicarbonate (ABC) and 1% trifluoric acid (TFA) were prepared beforehand. The entire alkylation procedure was performed inside a laminar flow hood. DTT stock solution was diluted 10 times with the protein sample and incubated for 15 min. The final volume was used to dilute IAA stock 10 times and the new mixture was incubated in dark for 30 min. Sample was then diluted with ABC stock solution three times. Then 0.2 µg trypsin was added to the solution. Samples were incubated overnight at 37°C. Finally, stock TFA solution was diluted 10 times with the sample volume. Sample was then cleaned of all contaminating salts with solid phase extraction with Thermo-Pierce

C18 Spin Columns protocol. Eluted samples were vacuum dried and stored in -20°C freezer for analysis.

2.5.LC-MS

Stored samples were resuspended with 10 µl of 0.1% formic acid (FA) and transferred to an LC-MS analysis vial. A reverse phase separation column that binds hydrophobic proteins was used in LC procedure which separates proteins depending on their hydrophobicity. For that purpose, LC was set to change from 100% water + 0.1% FA (Solution A) to 20% water + 80% acetonitrile (ACN) + 0.08% FA (Solution B) in one hour forming an exponential gradient change as seen in Table 1.

Table 2.1. Working conditions of the LC to separate proteins depending on their hydrophobicity

Retention time (min)	Flow (µl/min)	% Solution A	% Solution B
0	0,300	96.0	4.0
0	0,300	96.0	4.0
3	0,300	96.0	4.0
15	0,300	90.0	10.0
35	0,300	65.0	35.0
40	0,300	35.0	65.0
41	0,300	5.0	95.0
50	0,300	5.0	95.0
51	0,300	95.0	5.0
60	0,300	95.0	5.0

A Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer was coupled to the output of the LC instrument to separate proteins according to their mass and to identify them. All proteins with concentrations above identification threshold were taken for collision and identification by the instrument. The MS instrument was programmed so that the same mass proteins would not be selected for identification within a ten second window. After analysis, all data were converted from .raw to .mgfformat for faster bioinformatic analysis.

2.6. Bioinformatic Analysis

Initial bioinformatic analysis was performed with Progenesis QI (Dakna et al. 2009), a program that is specifically for analysis of LC-MS data. Initial statistical analysis as well as peak analysis were performed with this program.

Further bioinformatic analysis was performed by the Bioinformatics Group of Assoc. Prof. Dr. Jens Allmer in Izmir Institute of Technology. Analyses were done with a general setting for all programs. For modifications, deamination, carboxymethyl cysteine, carboamidomethyl cysteine, oxidation of methionine, phosphorylation of serine, phosphorylation of threonine and phosphorylation of tyrosine were taken into account during analysis. Fragmentation type was set to HCD. Precursor mass tolerance was set to 30 ppm as suggested by QExactive manual. Fragment (Product ion) tolerance was set to 0.2 dalton. For all searches, a self-constructed database including 236 *P. distans* and 10012 *P. tenuiflora* ESTs was used.

Three algorithms, OMSSA (Sadygov et al. 2009), MSDFDB and pFind (Fu et al. 2004) were used as search algorithms and hits of each algorithm were compared to each other to select the most reliable candidates. For OMSSA, ion types to be searched was set to a, b and y. Miscleavage was set to 2, E-value was set to 10^{12}, and number of hits per spectrum was set to 10. For MSDFDB, QExactive and HCD were selected. Therefore, the algorithm was adjusted for fragment tolerance and the ion types to be searched. Number of hits per spectrum was set to 10. The third program, pFind was run with minimum peptide length settings set between six as minimum and sixty as maximum. Miscleavage was set to 2 and instrument type was set to ESI-Trap CID since there were no settings for HCD.

Sequence identified peptides were subjected to the database search where the peptides were mapped to ESTs. Identified EST sequences were then searched in National Center for Biotechnology Information (NCBI) BLAST (Madden 2013) to find similar genes. Finally, these genes were run in BLAST2GO (Conesa and Götz 2008) program to identify gene ontology functions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Plant Growth and Tissue Boron Concentration

P. distans was observed to have extreme tolerance to high concentrations of boron. Shown in Figure 3.1, the differences can be seen between stress and control groups. Aside from chlorosis on the leaf tips and rigidification (Data not shown) of the leaf structure, the stress group did not show any other symptoms or necrosis in any tissues. Rigidification of leaves was expected since boron is an important element in synthesis of cell walls and forms crosslinks with pectins (Brown et al. 2002). Crosslinking of boron with pectins shrinks the size of cell wall pores which increases the wall's strength. In contrast, boron deficient plants were observed to have leaves with abnormal thicknesses (Matoh et al. 2000)

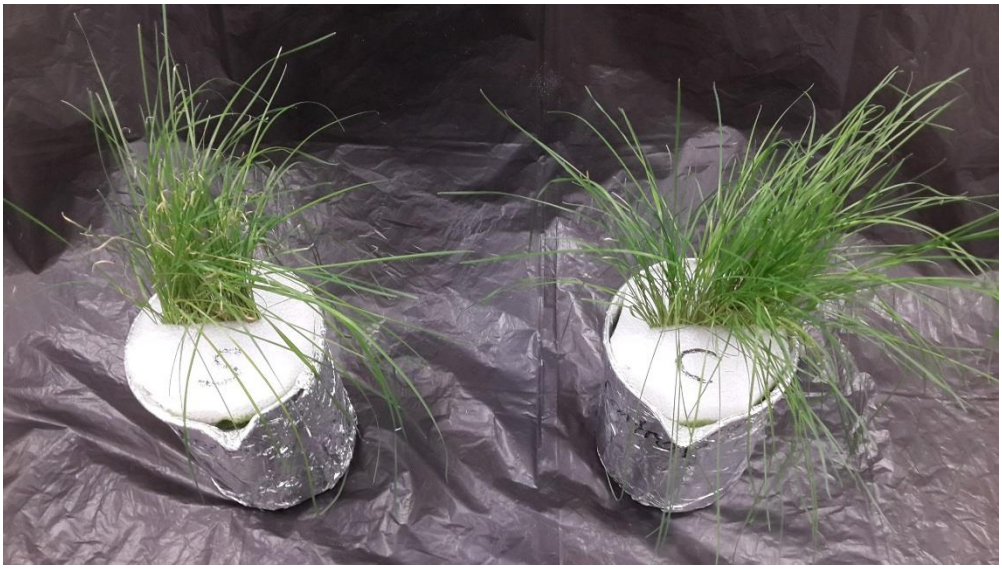

Figure 3.1. *P.distans* stress (left) and control (right) phenotypes after 2 weeks of stress treatment

Leaf boron concentration measurements through ICP-MS showed even higher amounts than previously observed by Babaoğlu et al. (2003). In Table 3.1, observed and previously published data are compared. As can be seen, there was more than eight fold difference between the results of boron accumulation in leaf tissues from samples

collected from nature and samples treated with boron in the lab. This result shows us that *P. distans* has even greater capacity to accumulate boron in its tissues without adverse phenotypic effects than was observed in nature. This property of *P. distans* makes it a perfect candidate for this work and any future work concerning boron phytoremediation in agricultural areas.

Table 3.1. *P. distans* tissue boron concentration results from ICP-MS after two weeks of stress treatment.

P. distans Boron accumulation results from ICP-MS		
B (µg/g)	Root (µg/g)	Leaf (µg/g)
Control (0.5 mg B/L)	137,8	127,2
Stress (500 mg B/L)	6161,0	6526,0
P. distans Boron accumulation results by Babaoğlu et al. 2003		
From natural environment (B conc. unknown)	241,0	802,0

3.2. Protein Extraction and LC-MS Analysis

The protein extraction method followed in this work was specifically designed to extract high quality proteins without detergents like CHAPS in the end product. Such detergent affects the results of liquid chromatography and in turn gives vertical streaks in an LC-MS profile.

The LC-MS profiles obtained from the instrument showed good reproducibility between repeats of the samples. There was no detergent interference or protease contamination since there were no vertical or horizontal streaks present. Figure 3.2 and Figure 3.3 show the LC-MS profiles of leaves of control and stress groups.

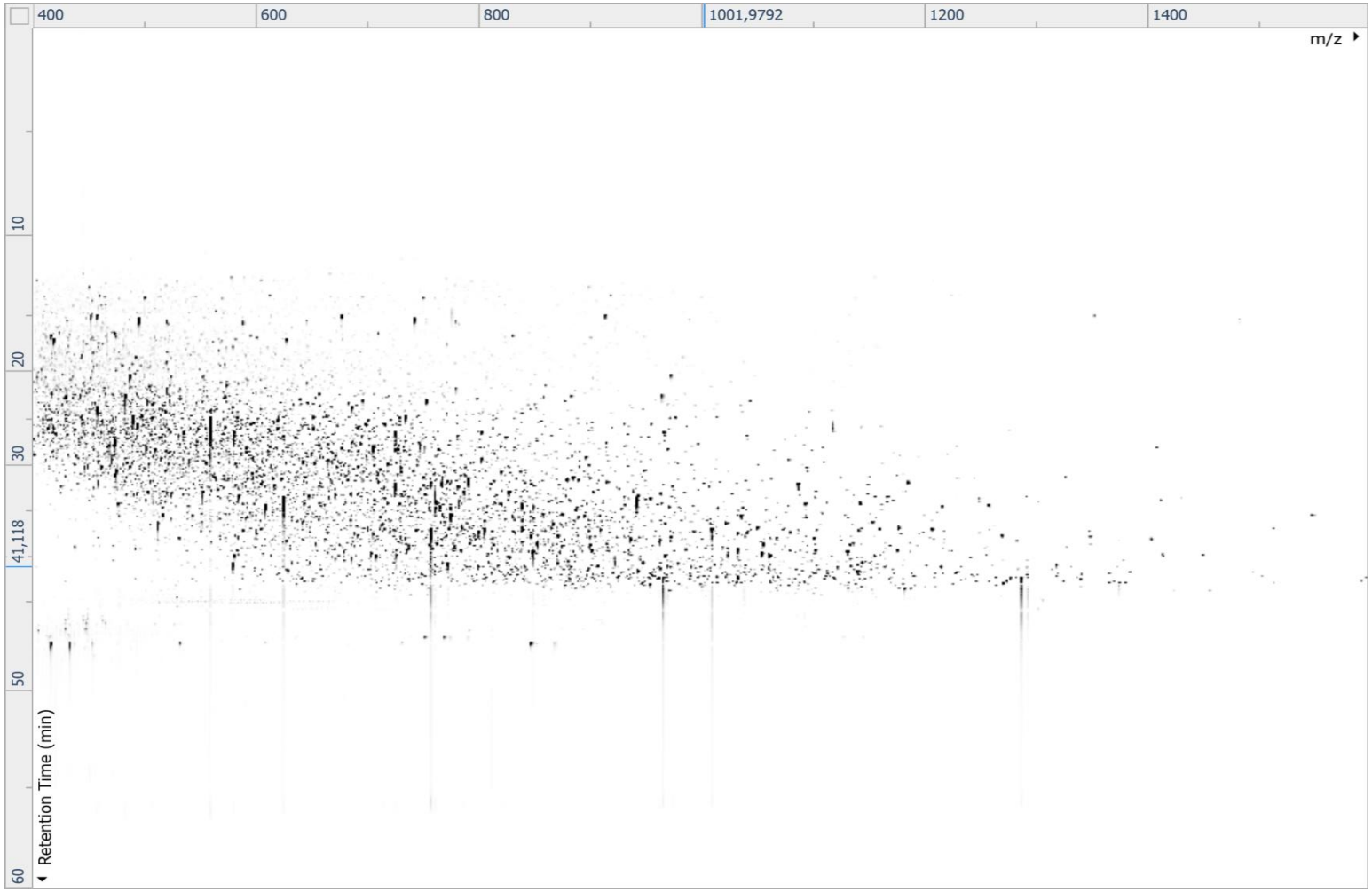

Figure 3.2. LC-MS protein profile of leaf tissue of control group.

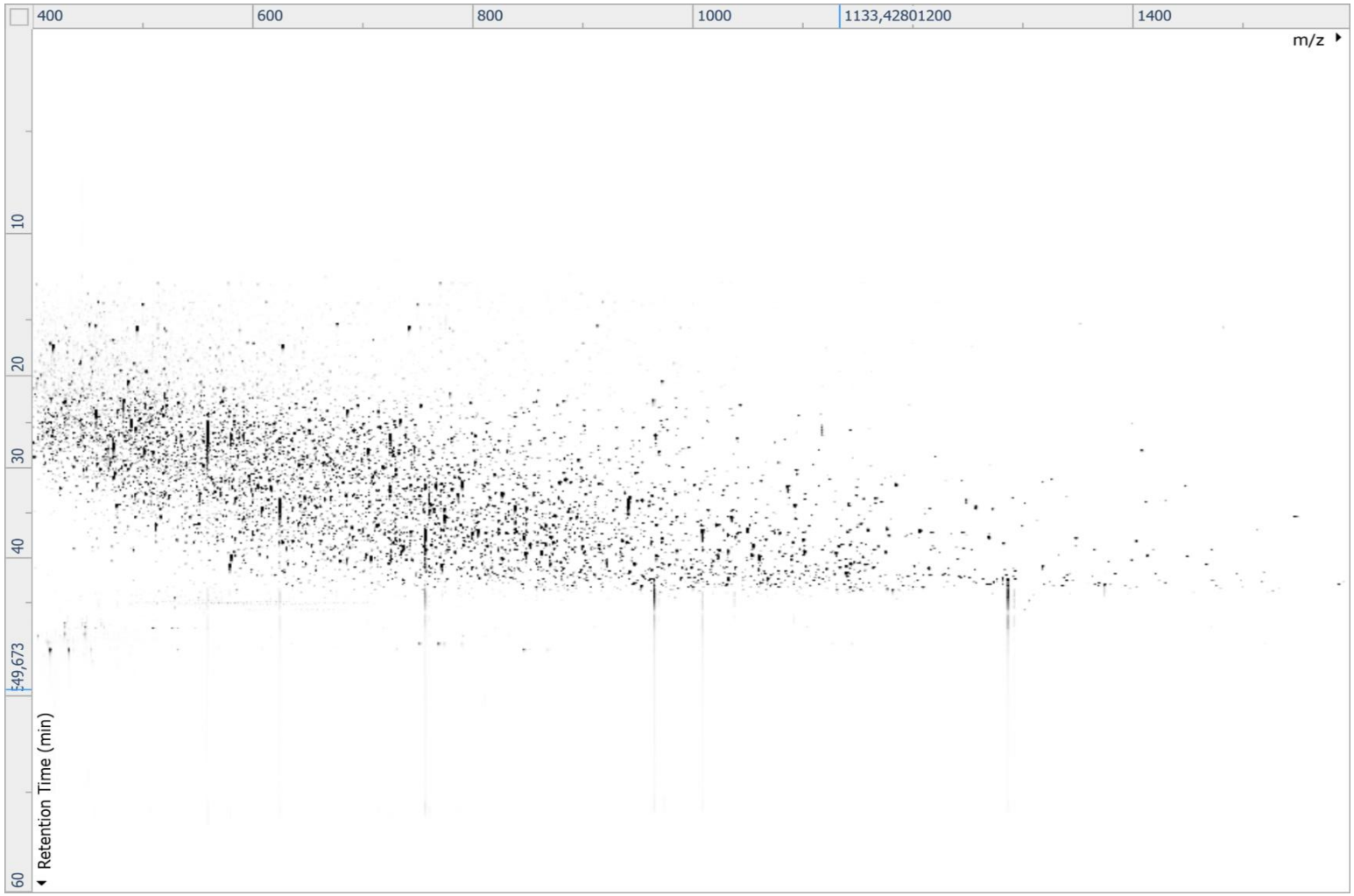

Figure 3.3. LC-MS protein profile of leaf tissue of stress treatment.

As seen from the LC-MS profiles, the proteins obtained from LC column were within around 10 and 50 minutes which is the time range during which the hydrophobicity of the mobile phase was increased with the 50th min being the most hydrophobic moment of the mobile phase. A wide range of masses was observed from leaf tissues ranging from 500 m/z to 1500 m/z. Total number of spectra obtained was similar for the first and second replicates, however, the third replicate showed around two thousand differences for leaves and one thousand differences for stress samples (Table 3.2).

3.3. Bioinformatic Analysis

A total of 183009 spectra was obtained from LC-MS analysis where the sequences of the peptides were identified by three algorithms with an overall coverage of 97.5%. In Table 3.2 the number of spectra obtained for each replicate and the identification rates are given.

Table 3.2. Table of total and identified number of spectra after LC-MS and Bioinformatic analysis, respectively.

Source Spectra	Total Spectra	Identified Spectra	Unidentified Spectra	Identified Spectra (%)	Unidentified Spectra (%)
CL1	31729	31071	658	97.9	2.1
CL2	31770	31123	647	98.0	2.0
CL3	29661	28830	831	97.2	2.8
SL1	30263	29505	758	97.5	2.5
SL2	30171	29279	892	97.0	3.0
SL3	29415	28558	857	97.1	2.9
Total	183009	178366	4643	97.5	2.5

Three different algorithms were used for peptide sequence identification in order to decrease the possibility of misidentifications. Shown in Figure 3.4, the spectra identified by the three algorithms individually and all together are shown. As can be seen, a high percentage, 72.5 %, of all the spectra were identified by all three

algorithms. This shows that the spectral identifications supported each other and were reliable for the EST database search.

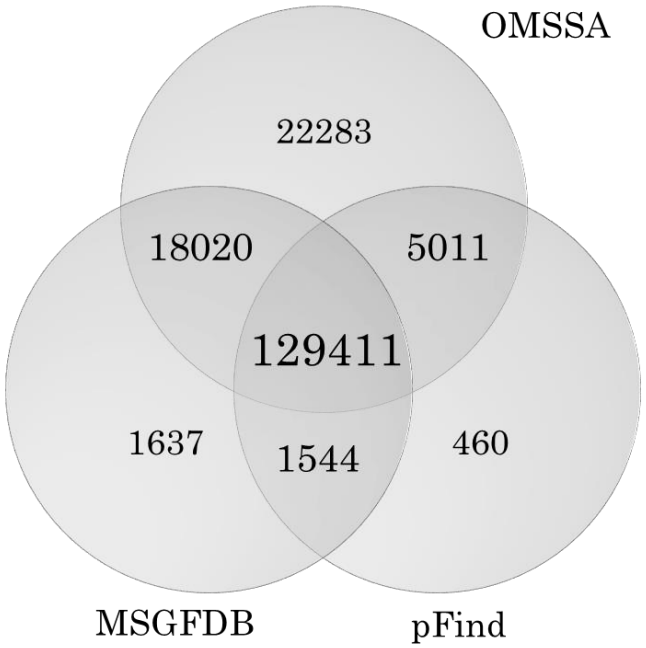

Figure 3.4. Numbers of spectra identified by each program.

Peptides with identified sequences were then selected for uniqueness since there were multiple hits of spectra per peptide sequence. Multiple hits can be caused by repeated analysis of the same protein in LC-MS due to a long isolation time from the hydrophobicity column. The LC-MS is calibrated so that it does not analyse the same protein twice in ten seconds. However, if a protein is eluted for longer than ten seconds and is still above the threshold, LC-MS will take it for another analysis creating the same spectrum with a different name. As a result of this reduction 13468 unique peptides were found to be supported by all three algorithms, 8627 were found to be supported by two and 156270 peptides were supported by only one program as shown in Table 3.3.

Table 3.3. Number of unique peptide sequences identified by how many programs.

Number of Tools Supporting Identity	Identified Unique Peptides
1	156270
2	8627
3	13468

To keep a broader scope for the research, all peptides with unique identified seqences were taken for EST database search. A total of 213931 database matches for all peptides were identified. Given that the number was higher than the searched peptides, the cause was investigated. It was found that more than one peptide was found to correspond to one EST sequence annotated in NCBI with high spectra support. This means that large proteins were divided into small parts within the collision cell of the mass spectrometer and these parts indicate the presence of the same protein during analysis. These EST sequences were annotated from the work of Wang et al. (2007) and Zhang et al. (2013) who studied the molecular features of the alkaline stress tolerance of *P. tenuiflora*.

Spectra support of all EST matches from all replicates of control and stress groups were then internally pooled and the general distribution was observed (Figure 3.5). The spectra support for the major part of identified EST sequences reside in the range of 1 and 39 for control and 1 and 33 for stress group. With outliers having up to 200 spectra supporting the sequences. There were eleven EST sequences with no spectra support from stress group and low spectra support from control group. Even though these sequences seem to be only expressed under normal conditions, the low number of spectra from control groupLC-MS analysis cannot support the claim. In order to analyze the differential expression of proteins between control and stress groups, a threshold value was set to exclude sequences with low spectral support. Since the control samples had an overall higher spectral support, the minimum spectral support threshold was set to five, while the threshold for the stress group was set to three.

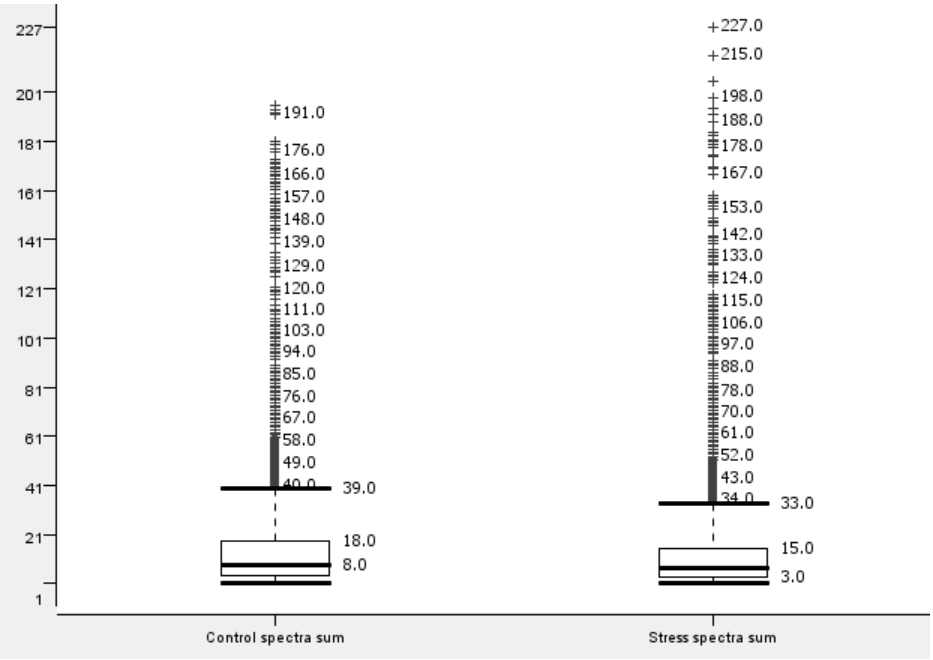

Figure 3.5. General distribution of spectra support of all identified control and stress EST sequences.

Using these thresholds, the EST sequences were filtered. Stress spectra counts of filtered EST sequences were divided by the control spectra counts which yielded the regulation fold difference under stress conditions. After division, another filtration was performed to separate proteins that are expressed similarly under control and stress conditions. As a result, 189 proteins were identified to be significantly differentially expressed with 163 of these proteins downregulated (Table A.1.) and 26 upregulated (Table B.1.). A gene ontology search performed with these differentially expressed proteins allowed a general understanding of the functions of these proteins. While 24 (92%) upregulated proteins were identified with GO functions, this number was 153 (94%) for downregulated proteins.

Downregulated proteins consisted of general cell metabolism proteins and ribonucleoproteins while among the upregulated proteins there were stress tolerance related proteins. In a scenario where boron is accumulated and tolerated within the cell, it is expected that certain proteins should be upregulated rather than downregulated to prevent boron toxicity. For this reason, upregulated proteins were investigated in a more comprehensive manner. With further investigation of each upregulated protein, three candidate proteins were chosen that may contribute to boron hyperaccumulation and tolerance in *P. distans*.

The first of these three proteins is a ferritin like protein with accession number GW405414. A ferritin protein takes role in iron storage, transport within the cell as well as maintaining cellular iron ion homeostasis (Theil 1987). They are large globular proteins with 24, 4-helix monomers (Marchetti et al. 2009). Within the 4-helix monomers of ferritin protein, Fe (III) ions are stored as ferric oxide mineral. A ferritin molecule can store up to 4.500 Fe (III) ions and these ions can be released depending on the concentration fluxes within the cell to maintain cellular homeostasis (Liu and Theil 2005). It is possible that the identified ferritin like protein stores boron which is a 3A group element as it may also have three valencies. Such a mechanism might be the backbone of the hyperaccumulation property of *P. distans*.

Another candidate protein was identified to be glyoxosomal malate synthase like protein (GMSLP) with an accession number of CN487673. As the name suggests, it takes part in malate synthesis in glyoxosomes, a specialized plant peroxisome. Malate is an important organic acid since it plays an important role in plant nutrition by exudation from the roots and solubilization of insoluble phosphorus and iron in the environment (Schulze et al. 2002). Besides nutrition, malate is also an important factor in aluminum tolerance. When malate is released from the roots, it chelates aluminum ions, causing them to precipitate, decreasing aluminum toxicity (Cocker et al. 1998). Boron is found in soil in both soluble and insoluble forms (Nable 1997). Goldberg (1997) found that boron could be adsorbed on aluminum oxide under 7-9 pH levels. Depending on the results of the experiment it is possible that boron uptake may be related to aluminum uptake, as well, which would not only cause boron toxicity but also aluminum toxicity to the plant. However by release of malate to the environment, both pH is reduced and aluminum is chelated where boron cannot be adsorbed onto aluminum oxide and the toxic effects reduced.

The third and last candidate protein for boron hyperaccumulation and tolerance is a thioredoxin h-type protein with the accession number JZ105491. These proteins are small ubiquitous proteins that are found in a wide range of species (Gelhaye et al. 2004). In general thioredoxins function as redox carriers in physiological processes like DNA synthesis, sulfur assimilation, and regulation of transcription factors (Dos Santos and Ray 2006). The identified thioredoxin h-type proteins specifically take part in oxidative stress and act as electron donors to other antioxidant proteins such as, peroxiredoxin, methionine sulfide reductase and glutathione reductase (Gelhaye et al. 2004). They are localized in the cytosol and mitochondria where most reactive oxygen

species are produced and help to prevent oxidative damage (Dos Santos and Ray 2006). Since boron stress produces high amount of reactive oxygen species and thioredoxin h-type proteins have antioxidant properties to prevent oxidative damage, it is highly possible that these proteins are closely related to the reduced toxicity of hyperaccumulated boron within the cell.

CHAPTER 4

CONCLUSION

P. distans plants were proven to show high tolerance to boron while accumulating it in toxic concentrations within their tissues. As observed at the end of their stress treatment, the leaves of stress groups were more rigid than the control group (data not presented). This observation suggests that boron might be stored in the cell wall as a structural unit thereby reducing its toxic effects.

Proteins were successfully isolated with high purity and run successfully with LC-MS instrumentation. Analysis of the results obtained from LC-MS procedure indicated that many proteins found in *P. distans* under boron stress were also identified in alkaline stress treated *P. tenuiflora* as they were present in the EST database.

A total of 189 significantly differential expressed proteins were identified with 26 upregulated and 163 downregulated proteins. GO functions of 177 of these identified proteins were found and three candidate: Ferritin-like protein, GMSLP and thioredoxin h-type protein were selected as the most likely to be involved in the tolerance and hyperaccumulation mechanism of *P. distans*.

In light of selected candidate proteins, further investigations should be made to understand the sequestration of boron to determine if boron is chelated, sequestered or stored within the cell wall as a structural unit. Also further studies should be performed to understand the role of ferritin or ferritin like protein in boron accumulation. The role of GMSLP is also an interesting concept to be investigated since by that mechanism availability of several minerals is changed in soil. If it is found that this mechanism allows the plant to solubilize and use the insoluble boron, *P. distans* may be used in phytoremediation studies to clear boron contaminated soils.

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APPENDIX A

TABLE OF DOWNREGULATED PROTEINS

Table A.1. Identified downregulated proteins with GO functions.

Gene Accession	Fold Regulation	Gene Ontology
GW404595	0.43	tpa: tubulin alpha-1 chain
GW405451	0.45	cdt1a protein isoform 1 isoform x1
GW405674	0.45	wall-associated receptor kinase 5-like
JK730972	0.38	germin-like protein 8-4
JZ098928	0.45	soluble inorganic pyrophosphatase
JZ099168	0.24	peroxisomal fatty acid beta-oxidation multifunctional protein
JZ099183	0.45	soluble inorganic pyrophosphatase
JZ099188	0.38	importin subunit alpha-1a
JZ099492	0.36	dead-box atp-dependent rna helicase 34
JZ099670	0.48	uncharacterized oxidoreductase chloroplastic-like
JZ099700	0.42	citrate synthase family protein
JZ099788	0.36	u3 small nucleolar rna-associated protein 14
JZ099894	0.35	bifunctional 3-dehydroquinate dehydratase shikimate partial
JZ100028	0.24	phospho-2-dehydro-3-deoxyheptonate aldolase chloroplastic-like
JZ100144	0.47	2-c-methyl-d-erythritol 4-phosphate chloroplastic-like
JZ100348	0.43	adp-ribosylation factor
JZ100594	0.45	pyruvate cytosolic isozyme

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Table A.1. (cont.)

JZ100613	0.43	adp-ribosylation factor
JZ099246	0.44	vesicle-associated protein 1-2-like
JZ101284	0.43	cell division control protein 48 homolog e-like
JZ101433	0.36	t-complex protein 1 subunit zeta
JZ101442	0.45	26s proteasome non-atpase regulatory subunit 3
JZ101690	0.33	serine threonine-protein phosphatase 2a 65 kda regulatory subunit a beta isoform-like isoform x1
JZ101773	0.46	jacalin-like lectin domain containing protein
JZ101818	0.38	udp-glucose 6-dehydrogenase
JZ101957	0.47	adenosine kinase 2-like
JZ102050	0.44	clathrin binding protein
JZ102156	0.38	5-methyltetrahydropteroyltriglutamate-homocysteine expressed
JZ102199	0.46	adp-ribosylation factor
JZ102259	0.40	glutathione s-transferase gstf2
JZ102267	0.44	dedicator of cytokinesis protein 7 isoform x1
JZ102296	0.40	ras-related protein rab7-like
JZ102347	0.29	transaldolase 2
JZ101009	0.40	rna polymerase ii degradation factor 1-like isoform x1
JZ101148	0.44	allantoate chloroplastic
JZ102404	0.40	glutathione s-transferase gstf2
JZ102482	0.45	calreticulin precursor
JZ102596	0.44	mannosyl-oligosaccharide glucosidase

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Table A.1. (cont.)

JZ102600	0.36	shikimate kinase
JZ102639	0.36	pra1 family protein b2-like
JZ102807	0.40	transmembrane 9 superfamily member 4-like
JZ102814	0.40	ras-related protein rab7
JZ103089	0.36	phosphoglycerate mutase-like
JZ103286	0.45	calreticulin precursor
JZ103334	0.43	pyruvate cytosolic isozyme
JZ103573	0.47	enoyl- hydratase peroxisomal-like
JZ103698	0.29	dihydrolipoyl dehydrogenase mitochondrial
JZ103700	0.44	duf21 domain-containing protein at2g14520-like
JZ103737	0.33	hat family dimerisation domain containing protein
JZ103753	0.40	dna excision repair protein ercc-6-like protein
JZ104019	0.33	chlorophyll a-b binding chloroplastic
JZ105762	0.43	mitochondrial-processing peptidase subunit beta
JZ105787	0.31	ribonuclease h protein
JZ105845	0.40	serine threonine kinase-like protein precursor
JZ104344	0.31	phospho-2-dehydro-3-deoxyheptonate aldolase chloroplastic-like
JZ104676	0.40	cytochrome b5 reductase 4
JZ104711	0.33	serine threonine-protein phosphatase 2a 65 kda regulatory subunit a beta isoform-like isoform x2
JZ105050	0.42	pentatricopeptide repeat-containing protein chloroplastic

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Table A.1. (cont.)

JZ105324	0.40	glucosidase 2 subunit beta
JZ105331	0.40	adenosine kinase 2-like
JZ105354	0.45	calreticulin precursor
JZ105899	0.47	udp-glucose pyrophosphorylase
JZ105967	0.24	nitrate-induced noi protein
JZ106125	0.37	enolase
JZ106498	0.44	retrotransposon ty3-gypsy subclass
JZ107239	0.41	glutamate-rich wd repeat-containing protein 1-like
CN485423	0.43	magnesium-chelatase subunit chloroplastic
CN485557	0.33	26s protease regulatory subunit 6a homolog
CN486669	0.44	ribosome-inactivating protein
CN486808	0.35	catalase
CN486905	0.35	catalase
CN487027	0.23	heme oxygenase 1
CN487126	0.40	ketol-acid chloroplastic-like
CN487141	0.33	bowman-birk type wound-induced protease inhibitor
CN487143	0.33	hypothetical protein LOC_Os10g18234
CN487211	0.48	thaumatin-like protein tlp5
CN487217	0.21	actin-depolymerizing factor 3
CN487461	0.29	protein nlp1 isoform x2
CN487692	0.31	alpha tubulin

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Table A.1. (cont.)

CN487715	0.39	proteasome subunit alpha type-5
EB104191	0.43	5-methyltetrahydropteroyltriglutamate-homocysteine expressed
EB104644	0.25	transaldolase 2
EB104828	0.36	chloroplast-localized ptr -binding protein1
EB105397	0.36	protein spiral1-like 1
EB105619	0.33	plastid-lipid-associated partial
Puccinellia Assembled_c18	0.44	snare domain containing protein
Puccinellia Assembled_c184	0.40	adenosine kinase 2-like
Puccinellia Assembled_c19	0.40	adenosine kinase 2-like
Puccinellia Assembled_c369	0.30	alanine aminotransferase
Puccinellia Assembled_c38	0.45	soluble inorganic pyrophosphatase
Puccinellia Assembled_c422	0.36	ketol-acid chloroplastic-like
Puccinellia Assembled_c428	0.43	adp-ribosylation factor
Puccinellia Assembled_c463	0.36	ras-related protein ric1
Puccinellia Assembled_c466	0.25	aldehyde dehydrogenase
Puccinellia Assembled_c47	0.44	syntaxin of plants 52
Puccinellia Assembled_c518	0.36	6-phosphogluconate decarboxylating

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Table A.1. (cont.)

Puccinellia Assembled_c558	0.36	aspartic proteinase
Puccinellia Assembled_c570	0.29	phenylalanine ammonia-lyase
Puccinellia Assembled_c596	0.45	soluble inorganic pyrophosphatase
Puccinellia Assembled_c623	0.25	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Puccinellia Assembled_c631	0.31	annexin a4
Puccinellia Assembled_c757	0.33	dehydroascorbate reductase
Puccinellia Assembled_c759	0.44	gtp-binding protein sar1a
Puccinellia Assembled_c779	0.40	protein early responsive to dehydration 15-like isoform x1
Puccinellia Assembled_c89	0.49	atp-citrate synthase beta chain protein 1-like
Puccinellia Assembled_c91	0.24	thioredoxin h-type

APPENDIX B

TABLE OF UPREGULATED PROTEINS

Table B.1. Identified upregulated proteins with GO functions

Gene Accession	Fold Regulation	Gene Ontology
GW405414	2.17	ferritin partial
JZ100179	2.50	golgi to er traffic protein 4 homolog
JZ100244	2.20	transmembrane 9 superfamily member 3-like
JZ101310	2.08	photosystem ii 10 kda polypeptide
JZ101315	2.57	hypothetical protein F775_29301
JZ102188	2.08	photosystem ii 10 kda polypeptide
JZ103874	2.08	photosystem ii 10 kda polypeptide
JZ105572	4.57	ferredoxin-dependent glutamate chloroplastic-like
JZ104194	2.50	transposon unclassified
JZ104399	2.08	photosystem ii 10 kda polypeptide
JZ104582	2.50	golgi to er traffic protein 4 homolog
JZ105074	2.67	h aca ribonucleoprotein complex subunit 4-like
JZ105491	2.29	thioredoxin h-type
JZ106779	3.33	morc family cw-type zinc finger protein 3-like
JZ107175	2.17	forminy 2 domain-containing expressed
CN485734	2.78	universal stress protein
CN486246	2.50	---NA---

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Table B.1. (cont.)

CN487271	2.38	---NA---
CN487304	2.08	photosystem ii 10 kda polypeptide
CN487673	2.30	malate glyoxysomal-like
EB104293	2.75	protein chloroplastic-like
Puccinellia Assembled_c170	3.83	ubiquitin-conjugating enzyme e2-17 kda
Puccinellia Assembled_c381	2.08	photosystem ii 10 kda polypeptide
Puccinellia Assembled_c64	2.08	photosystem ii 10 kda polypeptide
Puccinellia Assembled_c640	2.08	photosystem ii 10 kda polypeptide
Puccinellia Assembled_c788	2.08	photosystem ii 10 kda polypeptide