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Molecular Mechanisms of Boron Toxicity Tolerance in Plants

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MOLECULAR MECHANISMS OF BORON TOXICITY TOLERANCE IN PLANTS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in
The Department of Biological Sciences

by
Guannan Wang
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ABSTRACT

Boron toxicity is a worldwide agricultural problem that limits crop productivity and quality. However, our understanding on the genetic responses and adaption mechanisms to boron toxicity in plants is very limited. To address this gap in our knowledge, I compared boron stress-sensitive model, Arabidopsis thaliana and its stress-adapted relative Schrenkiella parvula to study how plants respond and adapt to excess boron at physiological, genomic, transcriptomic, and metabolic levels.

The overall project goal involved integration of multi-omics datasets to develop genome to phenome interpretations. To achieve this, I developed a python package, GOMCL, to facilitate the extraction of biologically meaningful information from transcriptomic data, and established an Agrobacterium-floral dip based transformation method for S. parvula to enable further functional characterization of candidate genes in this species.

Using a multi-omics framework with the tools developed, I demonstrated that excess boron induced pectin biosynthesis that facilitated boron sequestration in cell walls during excess boron stress, while the entire transcriptome shifted to a higher mean expression level. Compared to Arabidopsis, the magnitude of responses in S. parvula was much less. This was partly attributed to the greater capacity of S. parvula to maintain lower boron levels relative to A. thaliana. The transcriptomic analyses led to the identification of an understudied putative boron exporter BOR5, as the main candidate boron excluder during excess boron stress. We were able to characterize its boron exclusion function in yeast and show that SpBOR5 functioned more efficiently than any of the other closely related boron transporters in Arabidopsis and S. parvula. Besides, I showed that the S. parvula transcriptome is pre-adapted to boron toxicity, exhibiting substantial overlap with the boron-stressed transcriptome of A. thaliana.
In summary, I developed both computational tools and a transformation method to facilitate comparative genomic studies that use the extremophyte, *S. parvula* to study its stress adaptive mechanisms. With these tools, I investigated how excess boron can lead to cellular toxicity and how tolerant plants adapted to boron toxicity. The findings made during this investigation expands our current understanding of genetic responses underlying boron stress tolerance, while the methods developed during this project could be broadly applied in comparative genomics analyses.
CHAPTER 1: INTRODUCTION

Significance and conceptual framework

Global food security is challenged at unprecedented levels due to the increased demand from global population growth at present (Clay, 2011; Crist et al., 2017). This challenge is further compounded by the current climate crisis (Lesk et al., 2016; Pandey et al., 2017). Crop productivity loss due to excess boron, especially in arid and semi-arid areas, is among the leading challenges to our agriculture (Nable et al., 1997; Stangoulis and Reid, 2002). Boron toxicity often leads to the inhibition of plant growth and development by decreasing chlorophyll content, stomatal conductance, and photosynthesis, as well as by increasing ROS-mediated membrane damages, eventually resulting in premature death of shoots and roots (Lovatt and Bates, 1984; Karabal et al., 2003; Reid et al., 2004; Miwa et al., 2007). As many crops are sensitive to excess boron (Brdar-Jokanović, 2020) and the amelioration of high boron soils is extremely difficult (Nable et al., 1997), improving boron toxicity tolerance in crops becomes the most practical and effective way to counter excess boron-caused yield loss. To achieve this goal, it is necessary to understand the molecular mechanisms underlying this abiotic stress. However, despite extensive efforts to study boron toxicity responses in plants in the past, the molecular targets of excess boron and cellular and molecular processes interrupted by boron stress are poorly understood. Similarly, we have little understanding of the genetic mechanisms underlying boron toxicity responses or the adaptive mechanisms plants use to counter excess boron (Reid et al., 2004; Ruiz et al., 2003; Princi et al., 2016).

The ability of plants to tolerate toxic levels of boron varies with species (Landi et al., 2019). While the model species Arabidopsis thaliana is highly sensitive to excess boron, its closely related extremophyte model, Schrenkiella parvula (formerly Thellungiella parvula and Eutrema
S. parvula, in the Brassicaceae (Dassanayake et al., 2011; Zhu, 2015; Kazachkova et al., 2018), is tolerant to boron toxicity (Oh et al., 2014b). S. parvula can survive soils reported to have 5.8 mM boron (highly toxic to many plants) in its native habitats in the Central Anatolian Plateau, Turkey (Nilhan et al., 2008). Despite the striking differences in their adaptations to boron toxicity, S. parvula and A. thaliana are closely related species in Brassicaceae; have comparable life cycles; show similar growth rates and plant sizes; and have highly collinear genomes (Inan et al., 2004; Dassanayake et al., 2011). This presents an excellent system for comparative studies that can leverage the knowledge of molecular genetic studies conducted in A. thaliana. Therefore, the goal of this project is to first identify unique and shared responses in the two model species during excess boron stress at the ionomic, transcriptomic, and metabolic levels in association with their growth responses, and second to be able to deduce novel or enhanced functions observed in the extremophyte based on the A. thaliana knowledge base to be able to understand how plants may adapt to survive excess boron stress.

**Research objectives**

The overall goal of this project is to understand genetic mechanisms underlying plant responses to excess boron stress. To achieve this goal, I employed a multi-omics comparative approach using two model species, boron stress-sensitive A. thaliana and boron stress-tolerant S. parvula, and a number of computational and molecular tools (some of which were developed during this project).

I sought to address the following specific research questions: 1) how does excess boron creates a toxic cellular environment; 2) how does the two model species respond to excess boron differently; and 3) how does S. parvula achieve boron toxicity tolerance?
Summary of chapter layout

This dissertation is presented in 6 chapters that includes a literature review (Chapter 2), followed by research chapters (Chapters 3 to 5), and a general summary as a concluding chapter (Chapter 6). Chapter 2 has been formatted for submission in New Phytologist. Chapter 3 has been published in BMC Bioinformatics. Chapter 4 has been formatted for submission in The Plant Cell. Chapter 5 has been published in Journal of Visualized Experiments.

To provide an overview of the current understanding of boron stress responses and its effects in plants, a comprehensive literature review is presented as Chapter 2.

High throughput “omics” approaches often produce large lists of genes of interest. As a standard practice, pathway enrichment analysis is subsequently employed to determine the enriched functions among these differentially regulated genes based on Gene Ontology (GO) terms or other functional associations such as KEGG annotations (Ogata et al., 1999; Kanehisa et al., 2016, 2017). Even though this analysis significantly reduces the efforts required to mine the biological information represented by large groups of genes, the resulting enriched functions are still highly redundant making it challenging to identify true representative functions (Ashburner et al., 2000; Carbon et al., 2019). To address these limitations and summarize functional groups represented by large groups of genes, I developed the GOMCL toolkit presented in detail in Chapter 3.

Chapter 4 describes the multi-omics study developed to understand plant responses to excess boron stress using A. thaliana and S. parvula at physiological, genomic, transcriptomic, ionomic, and metabolomic comparisons. While excess boron induced drastic changes both at physiological and metabolic levels in A. thaliana, S. parvula remained largely unaffected when exposed to high levels of boron partly by maintaining a lower level of total boron and free boric
acid. Both species allocate significant transcriptomic and metabolomic resources to enable their cell walls to serve as a partial sink for excess boron. We provide evidence that the S. parvula transcriptome is pre-adapted to boron toxicity, exhibiting substantial overlap with the boron-stressed transcriptome of A. thaliana. Our transcriptomic and metabolomics data suggest that RNA metabolism is a primary target of boron toxicity. Cytoplasmic boric acid likely forms complexes with ribose and ribose-containing compounds critical to RNA and other primary metabolic functions.

Work described in Chapter 4 led to the identification of a candidate boron transporter that was previously functionally obscure but seemed to contribute to effective boron exclusion especially in the stress tolerant model. In the absence of an established transformation protocol for S. parvula, it was challenging to further investigate the role of novel genes from the extremophyte model. Therefore, Chapter 5 describes a modified Agrobacterium-mediated floral-dip method to generate stable S. parvula transgenic lines that enable functional investigations of genes of interest in this extremophyte.
CHAPTER 2: MOLECULAR MECHANISM OF PLANT RESPONSES AND ADAPTATIONS TO BORON TOXICITY

Introduction

As an essential nutrient, boron has the narrowest optimal range for plant growth and development (Eaton, 1944; Goldberg, 1997). Boron-rich soils are found in many agricultural lands across the globe, especially in arid and semi-arid areas (Nable et al., 1997; Stangoulis and Reid, 2002). When present in excess, boron is toxic to plants and adversely affects plant growth, resulting in losses in plant productivity and quality (Nable et al., 1997; Reid, 2013; Princi et al., 2016; Landi et al., 2019). There have been many more attempts to characterize plant responses to boron deficiency than to study how excess boron affects plants. Consequently, our understanding of the mechanisms behind boron toxicity is lagging behind our knowledge on how plants respond to boron deficiency.

Plant morphological and physiological responses to boron toxicity have been reviewed recently (Princi et al., 2016; Landi et al., 2019), especially in crops (Brdar-Jokanović, 2020; Chatzissavvidis and Antonopoulou, 2020). However, a comprehensive overview on boron toxicity responses in plants combining physiological responses to molecular level responses is lacking. In this review, we attempt to revisit the available literature on boron toxicity with a focus on molecular mechanisms underlying boron toxicity in plants, and how plants have evolved tolerance strategies to cope with excess boron.

Boron toxicity sources and distributions

Boron occurs widely in nature in various forms with an approximate concentration of 10 mg/kg in the earth’s crust and 4.6 mg/L in seawater (Woods, 1994; Argust, 1998; Princi et al., 2016; Brdar-Jokanović, 2020). It typically enters the environment mainly through weathering of sedimentary rocks, volatilization from oceans, volcanic activity, and, to a less extent, through
anthropogenic activities including irrigation malpractices (Howe, 1998; Stangoulis and Reid, 2002). The natural weathering of sedimentary rocks was suggested to be the predominant source of boron in soils and in the aquatic environment (Bertine and Goldberg, 1971). Total boron present in soils, the top layer of earth’s crust, range from 10 to 300 mg/kg with an average of 30 mg/kg (Howe, 1998; Padbhushan and Kumar, 2017). Soil boron can be divided into five groups: the readily soluble form in soil or weakly adsorbed by soil particles; the specifically bound form when adsorbed onto clay surfaces or associated with organic matter; the organically bound form if bound to organic matter; the oxide bound form if in association with oxides and hydroxides of Fe and Al; and residual boron forms when associated with primary and secondary minerals (Padbhushan and Kumar, 2017). Among them, only the readily soluble and specifically bound boron are available for plant uptake. The plant available boron accounts for 1-3% of the total soil boron content (Padbhushan and Kumar, 2017; Shah et al., 2017).

Soils formed from marine sediments or volcanic origins are inherently high in boron. These pose a major threat for crops in southern Australia, west Asia, and north Africa (Stangoulis and Reid, 2002; Yau and Ryan, 2008). The use of boron-rich irrigation water, excessive application of boron fertilizers, sewage contamination and surface mining runoff in agricultural fields, also increase soil boron to toxic levels to plants (Nable et al., 1997).

Boron toxicity is a world problem for crop yield and quality, especially in arid and semi-arid areas where leaching boron out from soil by rainfall or application of excessive water is not feasible (Nable et al., 1997; Stangoulis and Reid, 2002). Soils rich in boron have been reported in countries from all continents we grow plants, including Australia, Mexico, United States, Peru, Chile, Russia, India, Malaysia, Pakistan, Turkey, Israel, Syria, Jordan, Iraq, Hungary, Serbia,
Italy, Egypt, Morocco, and Libya (Nable et al., 1997; Stangoulis and Reid, 2002; Tanaka and Fujiwara, 2008; Yau and Ryan, 2008; Landi et al., 2019).

**Boron transport in plants**

Boric acid is a weak acid with a pKa of 9.24. At pH of 7.4, as found in cytoplasm, over 98% of boron is present in the form of free undissociated boric acid (H$_3$BO$_3$) and less than 2% is present as borate B(OH)$_4^-$ (Woods, 1996; Broadley et al., 2012; Princi et al., 2016). Free boric acid accounts for more than 99.95% of boron at lower pHs, for example, pH 5.5 found in the apoplast (Woods, 1996; Broadley et al., 2012; Princi et al., 2016). Thus, under typical soil conditions (pH 5.5 -7.5), boron predominantly exists as free boric acid and it is the major form of boron taken up by plants (Raven, 1980; Hu and Brown, 1997; Camacho-cristóbal et al., 2008). Boric acid is an uncharged small molecule with a molecular volume of 71.5Å$^3$, similar to urea (75.3Å$^3$) and other small nonelectrolytes (Dordas et al., 2000; Dordas and Brown, 2000).

The permeability coefficient of plant cell membranes to boric acid was calculated to be at least 10$^{-6}$ cm/s (Raven, 1980). However, the empirical permeability values were much smaller reported for squash roots (Dordas et al., 2000) and green algal species Chara (Stangoulis et al., 2001). Subsequent studies in tobacco and canola have indicated that passive permeation of boron was adequate to satisfy the boron requirement of plant growth under normal boron supply (Brown et al., 2002). Therefore, passive entry of boron is thought to be the primary mechanism of boron uptake in vascular plants when there is sufficient boron in the growth medium (Hu and Brown, 1997; Brown et al., 2002; Dannel et al., 2002; Tanaka and Fujiwara, 2008). Active boron uptake mechanisms are used by plants under limited boron conditions when passive boron uptake alone is insufficient to meet the demand in plants (Dannel et al., 1997; Pfeffer et al., 1997; Dannel et al., 2000; Stangoulis et al., 2001; Brown et al., 2002). To date, multiple boron
transporters have been characterized in plants (Reid, 2014; Yoshinari and Takano, 2017). Three mechanisms of boron uptake and transport in plants have been established (Figure 2.1): 1) passive diffusion across lipid membrane; 2) active transport by boron transporters; and 3) facilitated transport by boron transport channels (Tanaka and Fujiwara, 2008; Miwa et al., 2010; Princi et al., 2016; Landi et al., 2019). Once absorbed by the roots, boron can be transported to the shoots via the xylem powered by the transpiration stream (Brown and Shelp, 1997; Brown et al., 2002). While long-distance transport via xylem remains the dominant route, boron can be transported via phloem when bound to polyols (e.g. sorbitol, mannitol, dulcitol) or sucrose (Brown and Hu, 1996; Hu et al., 1997; Stangoulis et al., 2010).

Figure 2.1. Three modes of boric acid transport. (A) Transport via passive diffusion of boric acid; (B) facilitated transport of boric acid via boric acid channels; (C) active transport of boric acid via BORon transporters (BORs).

Boron uptake and translocation is regulated by two transmembrane protein families: boron transporter BOR family and major intrinsic protein (MIP) channels (Figure 2.2). There are seven members in the BOR family in the Arabidopsis genome (Takano et al., 2002; Miwa et al., 2010). BOR orthologs from other plants have been identified and grouped further into two main subfamilies: BOR1-like and BOR4-like family (Wakuta et al., 2015; Diehn et al., 2019). All BORs characterized to date are borate exporters (Reid, 2014; Wakuta et al., 2015). However,
these have been assigned different physiological functions based on the cellular localization (Hrmova et al., 2020).

![Diagram of Boron Uptake and Transport](image)

Figure 2.2. Boron uptake and transport from soil to roots during low, optimal, and high external boron levels. Under low external boron conditions, passive diffusion of boron is not sufficient to meet the plant boron demand. NIP5;1, BOR1, and BOR2 facilitate the uptake and radial transport of boron towards the stele from the epidermis. Under optimal conditions, boron is primarily taken up via passive diffusion, which is sufficient for maintaining plant growth. Under excess boron conditions, BOR4 exports boron back to the soil.

**Boron transporters**

The first identified boron transporter, *AtBOR1*, is preferentially expressed in roots, including the pericycle, columella, lateral root cap, epidermis and endodermis (Takano et al., 2002, 2010; Miwa et al., 2013; Yoshinari et al., 2016). Under boron-limited conditions, *AtBOR1* preferentially localizes to the stele-side of the plasma membrane in root cells through the AP2-dependent endocytic pathway (Łangowski et al., 2016; Yoshinari et al., 2019). This polar localization of *AtBOR1* facilitates the radial transport of boron towards the stele and efficient
loading of boron into xylem to be transported to the shoots (Takano et al., 2010; Yoshinari et al., 2016). Although \textit{AtBOR1} is expressed in shoots, its specific localization in shoots is unclear (Takano et al., 2002).

Under boron-limited conditions, the loss-of-function mutant of \textit{AtBOR1} showed severely reduced growth (Noguchi et al., 1997; Takano et al., 2001), while the overexpression of \textit{AtBOR1} improved plant growth and fertility (Miwa et al., 2006). Upon sufficient supply of external boron, \textit{AtBOR1} is trafficked from plasma membrane via the endosomes to the vacuole for degradation through boron-induced ubiquitination (Takano et al., 2005, 2010; Kasai et al., 2011). When the external boron further increases to toxic levels, \textit{AtBOR1} is translationally repressed by untranslated open reading frames (uORFs) present in its 5’-UTR (Aibara et al., 2018). This two-step repression of \textit{AtBOR1} ensures that \textit{AtBOR1} does not function under boron toxicity. The multiple uORFs found in \textit{AtBOR1}-type BORs suggest a conserved regulatory mechanism for BOR1 orthologs under excess boron (Aibara et al., 2018).

\textit{AtBOR2}, the closest paralog of \textit{AtBOR1} is also expressed in roots and is polarly distributed on the inner plasma membranes (Miwa et al., 2013). A significant proportion of \textit{AtBOR2} is localized to the secretory/recycling pathway between the plasma membrane and trans-Golgi network under limited boron conditions (Miwa et al., 2013). The loss of function mutant of \textit{AtBOR2} leads to a dramatic reduction of RG-II-B dimer formation in cell walls (Miwa et al., 2013).

\textit{AtBOR4} is the only boron transporter that has been demonstrated to function under boron toxicity. It is expressed in the epidermis of the root elongation zone, root meristem, and endodermis (Miwa et al., 2007, 2014). It is localized to the distal side of the plasma membrane (Miwa et al., 2007; Łangowski et al., 2010). \textit{AtBOR4} reduces excess boron influx into xylem.
and exports boron back to the soil to prevent boron accumulation in the plants under excess boron conditions (Miwa et al., 2007). The overexpression of \textit{AtBOR4} reduced \textit{in planta} boron concentrations, and improved plant growth under boron toxicity (Miwa et al., 2007). \textit{AtBOR4} loss of function mutant showed higher boron accumulation in shoots and decreased shoot growth in the presence of excess boron (Miwa et al., 2014). These studies demonstrated that \textit{AtBOR4} is a key determinant for boron toxicity tolerance in plants. \textit{AtBOR4} orthologs in barley and wheat have also been demonstrated to be critical for boron toxicity tolerance (Reid, 2007a; Sutton et al., 2007; Pallotta et al., 2014).

\textit{Boron channels}

\textit{NIP5;1} in Arabidopsis is the first aquaporin identified to facilitate boron uptake and transport in plants (Takano et al., 2006). It is localized to the plasma membrane of epidermal, cortical and endodermal cells in root elongation and maturation zones (Takano et al., 2006). When there is inadequate boron, \textit{AtNIP5;1} preferentially localizes to the outer plasma membrane domains, opposite to the polar localization of \textit{AtBOR1}, and is believed to mediate the radial transport of boron towards the stele in conjunction with \textit{AtBOR1} (Takano et al., 2010). Clathrin-mediated endocytosis maintains the polar localization of \textit{AtNIP5;1} (Wang et al., 2017). While boron deficiency induced \textit{AtNIP5;1} expression and translation, excess boron triggered 5’ UTR-dependent \textit{AtNIP5;1} mRNA degradation and translation inhibition (Tanaka et al., 2011, 2016). This post-transcriptional repression was caused by high boron-enhanced ribosome stalling at uORFs in the 5’ UTR of \textit{AtNIP5;1} (Tanaka et al., 2016). Loss of function mutations of \textit{AtNIP5;1} caused increased sensitivity to boron deficiency (Takano et al., 2006). Orthologs of \textit{AtNIP5;1} in maize and rice have shown similar supportive roles in boron transport required to alleviate boron deficiency symptoms (Leonard et al., 2014; Durbak et al., 2014; Hanaoka et al., 2014; Shao et al.,
Similar functional roles have been assigned to NIP6;1 and NIP7;1 under limited boron conditions (Tanaka et al., 2008; Routray et al., 2018). AtNIP6;1 is involved in xylem-phloem transfer of boron at the nodal regions and preferential boron transport to sink tissues (Tanaka et al., 2008). AtNIP7;1 is expressed in anther tapetal cells and facilitates pollen microspore cell wall formation (Routray et al., 2018).

AtTIP5;1, a member of tonoplast intrinsic protein (TIP) family, has been shown to enhance plant tolerance to boron toxicity (Pang et al., 2010). AtTIP5;1 is localized to the tonoplast and is therefore suggested to facilitate storing excess boron in vacuoles (Pang et al., 2010). Other aquaporins such as rice OsPIP1;3, OsPIP2;4, OsPIP2;6, and OsPIP2;7 are thought to function as bidirectional boron channels, and contribute to plant tolerance to boron toxicity (Kumar et al., 2014; Mosa et al., 2016).

**Boron toxicity symptoms at morphological level**

Boron transported via the xylem accumulates more in mature leaves as leaves normally represent the sites with the highest transpiration rates (Brown and Shelp, 1997). Consequently, boron toxicity symptoms are first observed in older mature leaves. It should be noted that boron in mature leaves can be translocated to younger tissue in certain plants by complexing with polyols and redistributed via the phloem (Brown and Hu, 1996; Hu et al., 1997; Brown and Hu, 1998a). In plants where boron is not redistributed via the phloem, the most common boron toxicity symptom is chlorosis followed by necrosis in mature leaves (Nable et al., 1997). Plants with parallel venation tends to show necrosis in the leaf tips first, whereas plants with reticulate venation show necrosis starting from leaf margins (Reid, 2013). The necrotic region spreads to the entire leaf surface and to the developing shoots with persistent toxicity (Brdar-Jokanović, 2020). Leaf cupping has also been reported as a specific symptom of boron toxicity in some
species (Loomis and Durst, 1992). Contrastingly, toxicity symptoms first appear in young stems and fruits in plants which use phloem transport to redistribute boron (e.g. *Prunus* spp., *Malus* spp., and *Pyrus* spp.). Toxicity symptoms in such plants have been often reported as fruit disorders, bark necrosis, and stem dieback (Brown and Hu, 1996).

Boron content in roots normally remains relatively low compared to that in shoots, especially in leaves, because boron is rapidly translocated to shoots from roots even at very high levels of external boron (Chatzissavvidis and Antonopoulou, 2020). Therefore, boron toxicity symptoms are rarely observed in roots (Nable et al., 1997). However, excess boron inhibits both primary and lateral root growth in many plants (Huang and Graham, 1990; LIU et al., 2000; Choi et al., 2007; Aquea et al., 2012).

**Boron toxicity mechanism at molecular level**

Figure 2.3 provides an overview of cellular processes and molecular functions affected during excess boron stress compared to growth optimal soil boron levels. Boron forms stable borate esters with *cis*-diols on a furanoid ring. Such structures are rare in nature and seem to be limited to apiose and ribose (Power and Woods, 1997). The only experimentally confirmed compound that binds to boron in plants *in vivo* is apiose in rhamnogalacturonan-II (RG-II) pectins found in cell walls (Ishii et al., 1999). Binding of boron to ribose and ribose-containing compounds has been shown *in vitro* studies (Pfeffer et al., 1999; Ralston and Hunt, 2001; Kim et al., 2003, 2004; Reid et al., 2004). Therefore, it has been long speculated that the main target candidates of boron toxicity are: 1) alteration of cell wall structure and development when excess boron binds to apiose in RG-II; 2) interruption of metabolic processes after excess boron binds to ribose in molecules such as adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD) and the reduced form (NADH) or nicotinamide adenine dinucleotide phosphate (NADP) and the
reduced form (NADPH); 3) disruption of cell division and development resulted from excess boron binding to ribose as the free sugar or within RNA (Stangoulis and Reid, 2002) (Figure 2.3).

Figure 2.3. An overview of plant responses to boron toxicity. Under sufficient and excess boron conditions, boron diffuses into plant roots. During sufficient boron conditions, absorbed boron cross-links newly synthesized RG-II and is exported to the cell wall. BOR1 and BOR2 are trafficked from plasma membrane via the endosomes to the vacuole for degradation. NIP5;1 is degraded mediated by the upstream open reading frames (uORFs). During excess boron conditions, BOR1 and BOR2 are trafficked from plasma membrane via the endosomes to the vacuole for degradation. BOR1 translation undergoes boron-dependent uORF-mediated inhibition. NIP5;1 is degraded and NIP5;1 translation is inhibited mediated by the uORFs. BOR4 and other BOR4-like family members export excess boron back to soil or into the apoplast. The increased intracellular boron bind to available RG-II molecules, resulting in increases in the proportion of RG-II-B monomers. Some of the excess boron is deposited into cell walls. The global transcription and translation increases. Polyhydroxyl metabolites needed for primary metabolism that could bind to boron are depleted. Intracellular reactive oxygen species (ROS) increases. Excess boron induces DNA damages mediated by excess boron-mediated histone hyperacetylation.
**Boron toxicity and cell wall**

Under normal conditions, > 90% of total boron in plants is localized to the cell wall (Loomis and Durst, 1992; Matoh et al., 1992; Hu and Brown, 1994; Blevins and Lukaszewski, 1998; Princi et al., 2016). However, it is unclear how the cell wall composition is affected under excess boron conditions. When Arabidopsis was exposed to excess boron, its concentration in shoots significantly increased with an enrichment of excess boron in non-cell cap fraction (Lamdan et al., 2012). A similar pattern was noted in the leaves from excess boron-treated citrus seedlings where the majority of increased boron content was found in two fractions: insoluble boron from cell wall and water-soluble boron from apoplast (Martínez-Cuenca et al., 2015). Consistent with these findings, a recent study found significant increases in boron concentrations in cell walls of excess boron treated Arabidopsis compared to control plants, especially in the shoots (Wang et al., 2020a). These indicate that cell wall is very likely a target of boron toxicity.

RG-II dimers account for ~95% of RG-II pectins in cell walls of Arabidopsis (O’Neill, 2001). Cross-linking of two RG-II molecules by borate occurs rapidly during RG-II synthesis and secretion in the cytoplasm. Borate does not seem to be able to cross-link the existing RG-II monomers in the cell wall (Chormova et al., 2014; Chormova and Fry, 2016). When monomerized RG-II was incubated with different levels of boric acid *in vitro*, RG-II dimerization was not inhibited by excess boron up to at least 50 mM (Chormova et al., 2014). Instead, the rate of RG-II dimer production appeared to be reduced as the external boron supply increased (Chormova et al., 2014). This inhibition on the rate of RG-II dimerization can be explained if excess boron depletes the pool of free RG-II monomers by rapidly complexing with available RG-II monomers. Previous studies have shown that excess boron increases the thickness of cell walls in the leaves of *Eriobotrya japonica* (Papadakis et al., 2018), *Poncirus*
trifoliata (Wu et al., 2018; Riaz et al., 2019; Wu et al., 2019), citrange (Citrus sinensis × Poncirus trifoliata) (Wu et al., 2018). It has been demonstrated that cell wall thickness of pumpkin leaves was inversely correlated with RG-II dimer formation (Ishii et al., 2001). The thickened cell wall under boron toxicity indicates a decrease in RG-II dimer content in the cell wall, which could result from a reduction in total RG-II content or an increased incorporation of RG-II monomers bound by excess boron in the cell walls. Recently, Wang et al. (2020) reported increased abundance in precursors of RG-II as well as an increases in the in transcripts leading to of RG-II biosynthesis genes in Arabidopsis treated with toxic levels of boron, suggesting that boron toxicity may elevate the total level of RG-II in the cell wall. Further, the ratio of RG-II dimers to monomers may decrease under boron toxicity (Figure 2.3).

As excess boron deposits in cell walls, it affects the expression of not only of genes associated with RG-II biosynthesis but also genes involved in the synthesis of other cell wall components. These include cellulose synthases and cellulose synthase-like family members. Notably, Cellulose Synthase Like-D5 (CSLD5) was found to be repressed by excess boron in Arabidopsis shoots (Wang et al., 2020a). CSLD5 has been shown to be critical for cell plate formation and subsequent plant cell divisions (Gu et al., 2016). The inhibition of CSLD5 in excess boron-stressed Arabidopsis may lead to cell division failures, which is especially problematic for meristematic tissues. Consistent with this, excess boron was demonstrated to decrease the number of mitotic cells and increased the fraction of 4C cells in Arabidopsis root tips (Sakamoto et al., 2011). On the contrary, excess boron does not affect percentages of cells in different cell cycle stages in human prostate and breast cancer cells (Barranco and Eckhert, 2004; Meacham et al., 2007; Bradke et al., 2008; Henderson et al., 2015). These suggest that excess boron might
exert toxic effects through inhibiting cell plate formation, which could eventually contribute to cell death in plants.

Boron toxicity induced the expression of suberin, lignin and cutin biosynthesis genes in treated Arabidopsis roots (Aquea et al., 2012). Further, Ghanati et al. (2002) have shown that cultured tobacco cells deposited more suberin and lignin into cell walls under excess boron conditions. This observation was accompanied by the increased enzymatic activities related to lignin biosynthesis. However, no significant reduction in viability of tobacco cells treated with excess boron was observed compared to those under control conditions, indicating that boron toxicity-induced changes in cell wall suberin and lignin are unlikely to cause cell death.

**Boron toxicity, transcription, and translation**

Binding of boron to ribose poses a threat to all ribose-containing compounds in the presence of excess boron. The complexation between boric acid and ribonucleotides has been investigated *in vitro* and shown to occur between boric acid and the *cis*-diols of the ribose moieties (Ralston and Hunt, 2001; Kim et al., 2004). Therefore, RNA is a potential target of boron toxicity. Using radiolabeled uridine and thymidine, Ali and Jarvis (1988) showed that RNA synthesis increased while DNA synthesis remained constant during external application of boron to mung bean hypocotyls. Additional studies on date palm and sorghum have shown increased RNA biosynthesis during excess boron conditions (Fawzia et al., 1994). A similar observation was made for human cell cultures as well (Dzondo-Gadet et al., 2002). Wang *et al.* (2020) reported that RNA metabolism was among the most affected cellular processes and the global average transcription expression level increased in Arabidopsis during excess boron stress. In agreement with this observation, Shomron and Ast demonstrated *in vitro* that boric acid could serve as a reversible regulator of pre-mRNA splicing in a dose-dependent way, stimulating splicing activity
between 5-10 mM while inhibiting it at higher concentrations (Shomron and Ast, 2003). This effect of high boron on splicing was shown to be independent of the interaction of boric acid with the exposed 3’ hydroxyl group on ribose (Shomron and Ast, 2003). It is unclear how excess boron stimulates RNA synthesis and splicing and if it affects RNA degradation.

The effect of excess boron on RNA translation is controversial. Toxic levels of boron decreased the amount of newly synthesized proteins as measured by the level of incorporated [35S]cysteine in cultured human dermal fibroblast (Benderdour et al., 1998) and [35S]methionine in yeast cells (Uluisik et al., 2011). This appears to be a result of boron stress-induced collapse in translation initiation or the direct inhibition of tRNA aminoacylation (Uluisik et al., 2011). Contradictorily, Dzondo-Gadet et al. (2002) demonstrated that boric acid acts as an activator for translation. However, these observations were not confirmed with any in vivo studies in plants. Boron toxicity does not cause any changes in the total leaf protein content in wheat (Wimmer et al., 2003), barley (Karabal et al., 2003), Arabidopsis (Chen et al., 2014), and citrus (Sang et al., 2015). When grown in [14C]glycine containing solutions, roots from boron stressed barley reduced the uptake of radiolabeled glycine while maintaining a steady state for protein synthesis, indicating that excess boron led to differential membrane transport of amino acid but not protein synthesis (Reid et al., 2004).

The steady state of amino acid abundances during excess boron stress may be governed by the species, tissue, and external boron concentrations. Medium to high boron stress did not change abundances of the majority of amino acids in barely shoots and roots (Roessner et al., 2006). Yet an increase in amino acid content was shown to be induced by boron toxicity in tomato leaves (Cervilla et al., 2009a) and Arabidopsis roots (Wang et al., 2020a). The divergence in the effect of excess boron on amino acid abundances can be attributed to the finding that excess boron
inhibits translation initiation and tRNA aminoacylation (Uluisik et al., 2011). Boron stressed plants may attempt to increase the amount of amino acids and translation to compensate for hampered protein synthesis (Figure 2.3).

*Boron toxicity and energy production*

Adenosine triphosphate (ATP) is a ribose-containing compound that is not only a genetic building block but is also the universal biological energy currency. ATP is one of the putative molecular targets of excess boron. To the best of our knowledge, ATP-borate complexes have not been isolated and confirmed *in vivo* to date. While several *in vitro* studies suggested a high affinity binding of boron to ATP (Pfeffer et al., 1999; Ralston and Hunt, 2001), Kim et al. reported that boric acid-ATP complexes were not observed when boric acid was incubated with ATP at alkaline conditions favorable for such interactions (Kim et al., 2004). To test the possibility of boron binding to ATP, Reid et al. (2004) examined the rate of protoplasmic streaming in *Chara corallina* cells, which was shown to be proportional to ATP concentration in the cell cytoplasm (Reid and Walker, 1983). They found protoplasmic streaming was initially unaffected at relatively low external boron levels and it significantly dropped when the boron concentration increased (Reid et al., 2004). Despite the supportive evidence for boron affecting the ATP level in *Chara*, angiosperm plant cells may have divergent responses to boron than what is deduced from a green alga. Unlike angiosperms, *Chara* cells can rapidly equilibrates with external boron conditions (Stangoulis et al., 2001). Therefore, compelling evidence is absent to decide that excess boron binds to ATP and depletes it from the metabolic pool.

Several other ribose-containing compounds, including NAD⁺, NADH, NADP⁺ and NADPH, are thought to be bound by boron. The complexation between boron and these nicotinamide nucleotides was demonstrated *in vitro* (Pfeffer et al., 1999; Ralston and Hunt, 2001; Kim et al.,
Boron binding affinity to these compounds is pH dependent and decreases in the order of NAD⁺, NADH, NADP⁺ and NADPH (Kim et al., 2003, 2004). Only the borate-NAD⁺ complex was observed at physiologically relevant pH (Kim et al., 2003, 2004). As a consequence, respiration (using NADH/NAD⁺), but not photosynthesis (using NADPH/NADP⁺) in barley leaves were inhibited by excess boron (Reid et al., 2004). However, only a small proportion of NAD⁺ is present as NAD⁺-borate esters at pH 7 to 8, leaving the majority of NAD⁺ free (Kim et al., 2003). Therefore, the significance of cellular toxicity caused by boron bound to NAD⁺ is not well established yet.

Boron toxicity and other processes

Previous studies report mixed results related to DNA damage caused by excess boron in animals. For example, excess boron was not found to cause DNA damage to certain human cell types (Robbins et al., 2010; Başaran et al., 2019) and fruit flies (Drosophila melanogaster) (Demir and Marcos, 2018). Contradictory results are however, reported from studies using human, rat, and zebrafish cells (Kim et al., 2009; Kitamura et al., 2012; Gülsoy et al., 2015). In plants, excess boron was found to increase the level of DNA damage in Arabidopsis (Sakamoto et al., 2011), wheat (Triticum aestivum L.) (Sahin et al., 2012), and maize (Zea mays L.) (Sakcali et al., 2015). Sakamoto et al. demonstrated that toxic levels of boron promotes the formation of double strand breaks (DSBs) in DNA from Arabidopsis root tip cells (Sakamoto et al., 2011). Recently, it has been shown that DNA damage caused by excess boron is mediated by boron-dependent histone hyperacetylation, and not by high boron-induced oxidative stress (Sakamoto et al., 2018). The histone hyperacetylation under boron toxicity leads to chromatin opening and destabilization, resulting in increased DNA susceptibility to damaging factors (Sakamoto et al., 2018). Sakamoto et al. (2018) further showed that 1 mM or higher boron levels promoted histone acetylation and
caused DSBs in cultured HeLa cells. This study identifies DNA damage as a major cause of excess boron toxicity (Figure 2.3).

Boron toxicity has been demonstrated to induce the production of reactive oxygen species (ROS), especially $\text{H}_2\text{O}_2$, and introduce membrane damages through lipid peroxidation (Molassiotis et al., 2006; Gunes et al., 2006; Cervilla et al., 2007; Eraslan et al., 2007; Wang et al., 2010; Pandey and Archana, 2013; Moustafa-Farag et al., 2020). Additionally, excess boron has been shown to specifically inhibit the formation of glutathione from cysteine in sunflower (Ruiz et al., 2003). It has been widely reported that excess boron exerts its toxic effects partially through oxidative damage. Often, boron stress induces many of the key antioxidant enzymes found in all land plants (Princi et al., 2016; Landi et al., 2019). The abundance of several non-enzymatic antioxidants, including ascorbate and glutathione are also shown to increase in response to excess boron (Cervilla et al., 2007; Landi et al., 2013a). Plants with relatively low ROS scavenging capacities tend to be more susceptible to boron toxicity compared to those that have enhanced ROS scavenging capacities (Ardic et al., 2009; Landi et al., 2013a, 2013b).

Therefore, the toxic effects of excess boron can be partially attributed to induced oxidative stress, which subsequently causes impairment of normal metabolic activities by damaging key cellular structures, as shown for many other stresses (Demidchik, 2015).

Excess boron affects the activities of enzymes from different metabolic pathways. As previously described, enzymes that depend on NAD$^+$ can be inhibited by excess boron (Reid et al., 2004). A similar inhibitory effect of excess boron has been observed for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and fructose-1,6-bisphosphatase (FBPase), α- and β-amylase, and root invertases (Han et al., 2009; Wang et al., 2016; Choi et al., 2007). Enzymes involved in the phenylpropanoid pathway were often induced during excess boron in many plant species.
This was often followed by increases in lignin and suberin and may not contribute to the toxicity effects of excess boron as discussed before. High levels of boron have also been reported to interfere with several enzymes that function in nitrogen assimilation. While excess boron tends to reduce the activities of nitrate reductase (NR) and nitrite reductase (NiR), it boosts the catalytic efficiency of glutamine synthase (GS), glutamate synthetase (GOGAT), and glutamate dehydrogenase (GDH) (Mahboobi et al., 2002; Cervilla et al., 2009a). This may indicate an elevated assimilation of ammonia, but not for nitrate in plants exposed to boron toxicity. In addition to these, some more enzymes have also been demonstrated to be inhibited by high boron, e.g. pectin methylesterases involved in cell wall remodeling (Wang et al., 2016), \( \gamma \)-glutamyl-cysteine synthetase and glutathione synthetase (Ruiz et al., 2003), and histone deacetylase (HDAC) (Sakamoto et al., 2018). Overall, accumulating evidence has suggested that some toxic effects of excess boron could be attributed to boron interference with enzyme activities.

**Boron toxicity tolerance mechanisms**

Three main mechanisms can be proposed for boron toxicity tolerance: (i) enhanced exclusion of excess boron from the plants; (ii) enrichment of excess boron in the apoplast; and (iii) improved intracellular tolerance of excess boron. These tolerance mechanisms are not mutually exclusive in tolerant plants.

*Exclusion of excess boron*

Plants that are more tolerant to excess boron stress have been reported to accumulate less boron levels compared to sensitive plants toxicity (Keleş et al., 2011; Xin and Huang, 2017; Simón-Grao et al., 2018; Kaur et al., 2006a; Wang et al., 2020a; Hamurcu et al., 2016). Studies that compared tolerant vs sensitive cultivars to excess boron stress in the same species have reported
the same trend (Nable, 1988; Hayes and Reid, 2004; Rehman et al., 2006; Reid, 2007a; Paull et al., 1991; Garnett et al., 1993; Bogacki et al., 2013; Kaur et al., 2006b, 2006a).

The lower internal excess boron accumulation in tolerant plants is likely achieved by the active efflux of excess boron (Hayes and Reid, 2004). As discussed in previous sections, the only known boron transporter that removes excess boron out of the plants under boron toxicity is BOR4 (Miwa et al., 2007). BOR-4 like members such as BOR5 and BOR7 have also been suggested to function in a similar way (Wang et al., 2020a) (Figure 2.3). BOR4, 5, and 7 are induced in Arabidopsis under excess boron conditions, while S. parvula (an extremophyte tolerant of excess boron stress) maintains higher basal transcript levels for these genes compared the orthologous basal expression observed in Arabidopsis (Wang et al., 2020a).

*Enrichment of excess boron in apoplast*

The ability to limit boron accumulation in tissues is not the only determinant of excess boron tolerance in plants. For example, rice cultivars that differed in their boron tolerance did not change in their boron accumulation levels (Ochiai et al., 2008; De Abreu Neto et al., 2017). A similar pattern was noted between sensitive and tolerant cultivars of barley (Torun et al., 2003; Atik et al., 2011) and chickpea (Ardic et al., 2009; Ardic et al., 2009).

Reid and Fitzpatrick demonstrated that in the tolerant barley cultivar Sahara, necrosis in leaves occurred at internal boron concentrations more than 3-fold higher than in the sensitive cultivar Schooner (Reid and Fitzpatrick, 2009a). However, the leaf protoplasts from Sahara had a lower cytoplasmic boron level compared to Schooner (Reid and Fitzpatrick, 2009a). The percentage of total boron eluted over 6 mins from leaf segments was significantly higher in the tolerant barley cultivar compared to the sensitive one under excess boron condition. These suggested that redistribution of boron from cytoplasm to the apoplast was prominent in the tolerant cultivar.
Transgenic Arabidopsis overexpressing *AtBOR4* in shoots show increased tolerance to excess boron compared to the wild type (Miwa and Fujiwara, 2011). When treated with 10 mM boron, leaf protoplasts of the transgenic line accumulated much less boron than wild plants. As proposed by Miwa and Fujiwara (2011), BOR4 enables the redistribution of excess boron from the cytoplasm to apoplast, alleviating cytoplasmic cytotoxicity. Supportive of the above study, an ortholog of *AtBOR4* in *Populus russkii*, *PrBOR7* expressed in leaves was among the highly induced *BORs* during excess boron treatments (Ou et al., 2019). The expression of a stabilized BOR1 variant, which escapes the high boron-induced degradation, in Arabidopsis was shown to increase plant tolerance to excess boron conditions despite increases in the total boron accumulated in shoots (Wakuta et al., 2016). As this modified variant of BOR1 is localized to the plasma membrane in leaves, it was suggested to increase boron tolerance in Arabidopsis by exporting boron from the cytoplasm to the apoplast (Wakuta et al., 2016). Additionally, the overexpression of bidirectional aquaporins *OsPIP1;3, OsPIP2;6, OsPIP2;4,* and *OsPIP2;7* in Arabidopsis also led to improved growth under boron toxicity without affecting the boron levels in shoots and roots (Kumar et al., 2014; Mosa et al., 2016). Taken together, these studies demonstrate the importance of excluding boron from the cytoplasm to achieve tolerance even when the excluded boron is in the apoplast and not necessarily extruded from the plant to the soil.

Several studies described before support the idea that free boric acid can be stored in the apoplast. More studies are needed to confirm this hypothesis. However, the observation that rainfall can mitigate the excess boron toxicity symptoms from barley leaves supports the idea that the apoplast holds free boric acid that can be leached from leaves (Reid and Fitzpatrick, 2009a). In addition, cell wall as discussed before can sequester boron in cell wall pectins.
However, cell wall sequestration of boron during excess boron treatments is variable and species specific. It has been suggested that cell wall does not seem to contribute effectively to the detoxification of excess boron in both roots and shoots of boron stress-resistant sunflower as there were only marginal increases in boron concentrations in the cell walls of the stressed plants (Dannel et al., 1998). Similar conclusion has been made for some tolerant Brassica rapa genotypes (Kaur et al., 2006a) and Schrenkiella parvula (Wang et al., 2020a). It seems plausible that excess boron is prevented from overaccumulating in cell walls in the resistant plants if cell wall participates in mediating toxic effects. Nonetheless, in these species, excluding excess boron from plants seems to be the primary strategy to combat excess boron, and it is not clear whether distributing excess boron into the apoplast plays a role in this process.

Intracellular tolerance of excess boron

Cytoplasmic tolerance of excess boron has been identified as a hallmark for several boron tolerant species (Keleş et al., 2011; Dannel et al., 1998; Dannel Frank, Pfeffer Heidrun, 1999; Kaur et al., 2006a; Lamdan et al., 2012; Wang et al., 2020a). Figure 2.3 provides an overview of cellular process that allows improved tolerance to cytoplasmic boron. Vacuole compartmentalization of boron is an effective way to avoid the accumulation of excessive boron in the cytosol as shown for many other plant toxins (Zhao et al., 2020; Sharma et al., 2016). However, sequestration of excess boron in vacuoles has not been clearly elucidated in plants. AtTIP5;1 is the only known boron facilitator that localizes to the tonoplast (Pang et al., 2010). Overexpression of AtTIP5;1 in Arabidopsis has led to enhanced boron toxicity tolerance, albeit the increases in the internal boron levels in shoots (Pang et al., 2010). It is hypothesized that AtTIP5;1 may decrease the cytoplasmic boron concentration by compartmentalizing excess boron into the vacuole to improve plant tolerance to boron toxicity. The citrus ortholog of
AtTIP5;1 was also found to be induced by excess boron in support of its role in alleviating cytoplasmic boron toxicity (Martínez-Cuenca et al., 2015). Contrastingly, the transcript abundance of AtTIP5;1 was recently reported to remain unaffected in stressed Arabidopsis (Wang et al., 2020a). Evidence for the role of vacuole in detoxifying excess boron it still limited and contradicting, and more direct experimental data will be needed to resolve the conflicts. When reaching similar intracellular levels of excess boron in shoots, Eutrema salsugineum showed tolerance to boron toxicity compared to its stress-sensitive relative Arabidopsis (Lamdan et al., 2012). A concurrent increase in polyhydroxyl metabolites, including malic acid, fructose, glucose, sucrose, and citric acid were observed to be associated with the boron tolerance capacity in E. salsugineum. These polyhydroxyl metabolites can potentially bind boron and is assumed to facilitate translocation via phloem. Lamdan et al. (2012) proposed that this mechanism of recirculating boron from shoots to roots allows the plants to extrude excess boron back into the soil enhancing boron toxicity tolerance in E. salsugineum. However, most species where boron is mobile in the phloem are also reported as more susceptible to boron toxicity (Nable et al., 1997). This may be due to the accumulation of excess boron if boron-polyol complexes are decoupled to meet the high demand for carbohydrates in developing tissue (Brown and Hu, 1996; Hu et al., 1997; Stangoulis et al., 2010). Further investigation is required to understand whether phloem recirculation contributes to boron toxicity tolerance in plants or compounds toxicity impacts. Based on previous studies, the role of antioxidants in boron toxicity tolerance is largely species or genotype dependent. A few studies argue that antioxidants do not play a significant role (Keleș et al., 2011; Karabal et al., 2003), but the majority suggest a significant role in achieving boron toxicity tolerance (Ardic et al., 2009; Landi et al., 2013b, 2013a; Hamurcu et al., 2016; Cervilla et al., 2007). Plants often associated with high boron toxicity tolerance are equipped
with strong antioxidant pools; show high constitutive levels of anthocyanin, glutathione, and ascorbic acid; and limit accumulation of ROS generating compounds such as H$_2$O$_2$. Several studies have also reported boron toxicity tolerance achieved through the induction of salicylic acid that led to the activation of antioxidant defenses (El-Shazoly et al., 2019; Moustafa-Farag et al., 2020). Exogenous treatments such as hydrogen gas and melatonin have shown to contribute to excess boron tolerance via limiting H$_2$O$_2$ production or increasing the antioxidant capacity (Wang et al., 2016; Al-Huqail et al., 2020). Collectively, there is strong support to suggest that antioxidants help plants cope with boron stress and achieve tolerance to excess boron.

Boron toxicity has been shown to induce histone hyperacetylation, which promotes chromatin opening and destabilization, and subsequently results in DNA damage (Sakamoto et al., 2011, 2018). This loosened chromatin state was suggested to be maintained by BRAHMA (BRM), a subunit of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex (Sakamoto et al., 2018). Sakamoto et al. (2018) have demonstrated that the degradation of BRM promotes boron toxicity tolerance in Arabidopsis by limiting excess boron-induced double strand breaks (Sakamoto et al., 2018). This mechanism allows plants to cope with excess boron tolerance by modulating chromatin stability and how plants use chromatin remodeling to achieve excess boron tolerance needs to be further explored.

Transcripts associated with RNA metabolism, boron transport, antioxidant defenses, cell cycle, and cell wall organization in the extremophyte Schrenkiaella parvula maintained at basal levels comparable to the boron stress induced states observed in Arabidopsis suggested strong support for pre-adapted transcriptional regulation at a genome-wide scale distinctly evolved in the stress adapted species (Wang et al., 2020a). Boron toxicity tolerance of sweet basil (*Ocimum basilicum*) cultivars was also proposed to be attributed to the higher basal levels of ascorbic acid,
glutathione and anthocyanins (Landi et al., 2013a, 2013b). Similar stress-primed constitutive expression has been reported for multiple other stresses (Taji et al., 2004; Becher et al., 2004a; Wong et al., 2006; Velasco et al., 2016; Oh et al., 2014b).

**Future perspectives**

Boron toxicity is a worldwide problem for agriculture, threatening the growth and production of crops. This problem could be worsened by the rising temperatures and changing precipitation patterns caused by climate change. Despite the extensive studies on plant response to excess boron, our understanding of the genetic mechanisms underlying boron toxicity tolerance is limited. Currently, little is known about how excess boron is sensed and signaled in plants. A growing body of evidence point to RNA synthesis and translation as major targets of cytoplasmic boron toxicity. Given that soil amelioration for boron rich soils is impractical, selection and development of boron toxicity tolerant crops could be the most effective way to overcome this agricultural problem. Although the advances in gene editing technologies has made the development of such tolerant crops more feasible, a better understanding of boron toxicity tolerance in plants will be imperative to develop novel boron tolerant crops. Plants that are naturally adapted to boron toxicity, such as *Puccinellia distans* and *Schrenkiella parvula*, represent an underexploited resource for identifying genetic innovations and regulatory networks that promote plant resilience under excess boron that might be absent in the sensitive plants. In addition, as boron toxicity often occurs in combination with salinity and drought, systematic investigations of the interactions between these stresses in the future will be critical to develop plants that can simultaneously cope with these stresses.
CHAPTER 3: GOMCL: A TOOLKIT TO CLUSTER, EVALUATE, AND EXTRACT NON-REdundant ASSOCIATIONS OF GENE ONTOLOGY-BASED FUNCTIONS*

Introduction

High-throughput “omics” approaches are frequently employed to investigate expression changes and regulation of genes at a genome-wide level. Use of these genomic data often results in the identification of large lists of genes of interest. A standard approach to summarize the functions of these genes is to determine the enriched functions represented by Gene Ontology (GO) terms and other functional associations extracted from databases such as KEGG (Ogata et al., 1999; Kanehisa et al., 2016, 2017), Reactome (Fabregat et al., 2018) and Pathway Commons (Cerami et al., 2011), known as pathway enrichment analysis (Khatri et al., 2012; Wadi et al., 2016). This approach significantly simplifies the need from understanding the biological meaning embedded in individual genes in a large list, to the interpretation of enriched gene sets that could serve as a summary of enriched functions.

GO resources have become the most widely used knowledge base in terms of gene functions (Ashburner et al., 2000; Carbon et al., 2019), which provides a controlled hierarchy of GO vocabularies describing biological processes, molecular functions, and cellular components. However, this hierarchical functional annotation system presents a high level of redundancy as parent GO terms include or partially overlap with child GO terms and one gene could be annotated with seemingly unrelated GO terms. The computational tool, Enrichment Map (Merico et al., 2010; Reimand et al., 2019) was initially developed to overcome this problem by building

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a GO similarity network built on the overlap between gene sets annotated with each GO term. Yet, the identification of GO clusters within the GO similarity network in Enrichment Map does not define clusters and therefore the user has to separate groups based on a visual selection, which can be heavily affected by the layout of the network visualizations. As a result, when there are large numbers of similar GO terms, it is challenging to identify significant functional groups using Enrichment Map. Another comparable tool, ClueGO, identifies functional groups by first creating all possible initial groups with a user-defined number of GO terms showing similarities equal or above the predefined threshold and then iteratively comparing and merging these initial groups if the overlap between them is above the predefined threshold (Bindea et al., 2009). However, ClueGO can assign unique GO terms to multiple groups, making it challenging to identify non-redundant clusters (Bindea et al., 2009). Additionally, this tool does not accept direct output files from other commonly used GO enrichment tools such as BiNGO, g:Profiler, or agriGO. Both tools fall short at parallel processing a large number of distinct set of gene functions often encountered in large-scale -omics experiments. To address these limitations and generate a similarity-based functional GO network, we developed a new toolkit, GOMCL, that applies the Markov Clustering (MCL) algorithm (van Dongen, 2000; Van Dongen, 2008; van Dongen and Abreu-Goodger, 2012) to identify cluster structures in GO networks in an unbiased approach. Each GO term is represented by a node and edges connect two GO terms that share a certain percentage of gene members in GOMCL. To further facilitate the interpretation of resulting functional groups, GOMCL allows users to generate hierarchy plots and provides sub-clustering options for any number of selected clusters. GOMCL is a user friendly python toolkit, which offers multiple visualization schemes and enables batch processing of large GO datasets to mine for functionally significant attributes.
Methods

GOMCL is implemented in Python and allows grouping of lists of individual GO terms of interest into GO clusters using MCL (Figure 3.1). GOMCL encapsulates its entire pipeline in a single command and offers default parameters with which users can expect optimal results.

**Input data.** The package accepts the direct outputs from a variety of commonly used GO enrichment analysis tools, including BiNGO (Maere et al., 2005), GOrilla (Eden et al., 2009), g:Profiler (Raudvere et al., 2019), and agriGO (Tian et al., 2017), as well as customized GO lists. Support for more enrichment tools will be provided. In addition to the GO lists, GOMCL requires a GO ontology file in OBO format from the Gene Ontology Consortium (http://geneontology.org/) as an input (Figure 3.1).

![Figure 3.1. The workflow of GOMCL clustering on GO enrichment test results.](image-url)
GOMCL workflow. GOMCL first trims the input GO lists by removing overly broad GO terms whose size is greater than a user-defined threshold. For example, a large GO term such as biological regulation (GO:0065007) has over 12,000 child GO terms, including 15,000 genes in Arabidopsis and is often uninformative as a term representing a meaningful biological function. GOMCL also enables users to separate input GO lists into biological process, molecular function, and cellular component categories or any combinations of these categories if clustering within different categories is preferred. Each term in the trimmed GO lists is then compared to each other, and similarity between any two GO terms is computed based on the overlaps between the members of these two GO terms as either a Jaccard Coefficient \((JC)\) or an Overlap Coefficient \((OC)\) (Merico et al., 2010). Given any two GO terms, \(A\) and \(B\), the Jaccard Coefficient \((JC)\) is calculated as \(A \cap B / A \cup B\), and preferred for clustering of similarly sized GO terms. The Overlap Coefficient \((OC)\) is derived from \(A \cap B / \min (A, B)\), and works better to maximally reduce the redundancy between disproportionately sized GO terms. The construction of the GO term similarity network is initiated using only those interactions that pass a user-defined threshold for the Jaccard or Overlap coefficient of users’ choice. MCL algorithm is subsequently applied to identify cluster structure in the initial network and assigns more similar GO terms into one cluster. The resulting GO clusters are ordered based on the number of genes in each cluster. GO terms with largest number of genes, or smallest enrichment p-value, or most other GO terms connected are selected and offered as potential representative GO terms for each cluster. GOMCL also reproduces the hierarchy of GO terms from the provided ontology structure for any user-selected clusters upon command to assist identification and interpretation of the functional themes of these clusters. A novel functionality enabled in GOMCL that is unavailable in previous tools for GO-network analysis, is the evaluation of clustering results by
visualizing the distribution of similarity indexes between GO terms for each cluster. Taking this one step further, GOMCL includes a second module, called GOMCL-sub, which provides users customizable options to break down selected clusters produced by GOMCL into sub-groups with more specific functional themes. These functionalities combined, allows users to determine if

Figure 3.2. Representative outputs created with GOMCL for clustering of enriched GO terms in a selected study to distinguish two cell populations (Wendrich et al. 2017). Overlap coefficient of 0.5 and cluster granularity of 1.5 were used in GOMCL for cluster identification. [A] Similarity heatmap, [B] network of identified GOMCL clusters. Node size represents the number of genes in the test set which are annotated to that GO term; edges represent the similarity index between GO terms; each cluster is coded with a different color; and shade of each node represents p-value assigned by the enrichment test. Lighter to darker shades indicate larger to smaller p-values, respectively. [C] A tabular summary of all GOMCL clusters. x: the number of genes in the test set; n: total number of genes in the reference annotation.
there are distinct functional themes present in primary clusters and further identify these substructures in clusters of interest.

**Output format.** The standard GOMCL output consists of a heatmap (Figure 3.2A), a graphical GO-similarity-network based on the clustering results (Figure 3.2B), a tabulation of each GO term with cluster information and depth information (Klopfenstein et al., 2018) from the provided ontology structure, and a summary file for all clusters (Figure 3.2C). Graphical presentations of similarity index distribution (Figure 3.3) and GO hierarchy for individual clusters (Figure 3.4) are generated if the user chooses that option to create additional result files. If the user plans to generate cluster depth information for each GO term and build GO hierarchies as an output file, we recommend that the same version of the GO ontology file used in the GO enrichment analysis tool where the GO input list is created to should be used as an input for GOMCL. In addition to the graphical outputs, the user can opt to generate simple
interaction format files with either similarity between GO terms or GO hierarchy (Supplement file 3.2*), both of which can be directly used as inputs, together with the information about clustered GO terms (Supplement file 3.1), to Cytoscape (Shannon, 2003) for further manipulation of GO network visualization.

Figure 3.4. GO hierarchical structure produced using GOMCL for cluster C1 described in Figure 3.1. Edges represent the parent/child relationships of the GO terms. The black edges connect parent and child terms that are directly linked, while the grey edges indicate connections with intermediate GO terms between the parent and child terms. Node size represents the number of genes in the test set which are annotated to that GO term; and shade of each node represents p-value assigned by the enrichment test. Lighter to darker shades indicate larger to smaller p-values, respectively. The main hierarchical branches are marked by red circles.

**Results**

As a proof of concept, we performed a GOMCL run on a list of over-represented GO terms identified from genes differentially expressed between two GFP tagged cell populations of Arabidopsis roots in a published study (Wendrich et al., 2017) to highlight the functional use of GOMCL. In this published study, a xylem-specific promoter was used to drive the expression of

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* All supplemental materials are available online with the original publication at journal’s website.
GFP in Arabidopsis, and root proximal meristem cells were later separated into two populations based on the intensity of GFP signals. Cells with high GFP signals were assumed to be close to the quiescent center while cells with low GFP signals were assumed to be located away from the quiescent center. A microarray analysis was then conducted to compare the two cell populations and the authors aimed to see a difference in gene expression associated with cell division between these two cell populations.

**Cluster identification.** We used GO terms that had less than 3500 genes annotated under each GO annotation for Arabidopsis, to allow identification of specific functional traits associated with the published study. This resulted in 244 total GO terms (out of 251) enriched in genes expressed higher in the cell population with high GFP intensity (Supplement file 3.1). The default *Overlap Coefficient* of 0.5 and granularity of 1.5 were used for cluster identification. These cutoffs can be set by the user. Among the 244 GO terms, GOMCL identified five distinct clusters with minimal overlap between clusters and extensive overlaps among GO terms within each cluster (Figure 3.2A). The largest cluster (C1) included 124 GO terms and was mainly related to developmental processes and reproduction (Figure 3.2B, C). The 4th largest cluster, albeit comprising only 33 genes from 20 GO terms (Figure 3.2B, C), was overrepresented in genes associated with regulation of cell cycle and was also found to be mostly associated with the largest cluster. These representative functional groups and associations reflected the essential difference in the growth stages between the high GFP cells (cells assumed to be close to the quiescent center) and low GFP cells (cells located away from the quiescent center) in the targeted study where it aimed to see a difference in gene expression associated with cell division between these two cell populations (Wendrich et al., 2017). The reduction from over 200 GO
terms to 5 GO clusters preserved the enriched functional themes and facilitated the explanation of major patterns identified among ~1000 differentially expressed genes.

**Cluster quality evaluation and sub-clustering process.** To demonstrate the use of cluster quality evaluation and sub-clustering, we first enabled options to generate similarity index distributions for all five clusters identified by GOMCL. As shown in Figure 3.3, the majority of similarity indexes between GO terms within cluster C3, C4, and C5 were greater than 0.5. However, there is a large proportion of GO terms from cluster C1 and C2 showing no or small overlaps with other GO terms from the same cluster. To assist determining whether cluster C1 and C2 should be further separated into groups with more specific functional themes, we plotted GO hierarchies for these two clusters with GOMCL parameter -hg on (Figure 3.4, Supplement file 3.3). Several distinct branches were identified from the hierarchical structures of GO terms within each cluster (Figure 3.4, Supplement file 3.3), indicating the possible presence of distinct sub-groups within these clusters. We further employed GOMCL-sub, which was designed to analyze selected clusters produced by GOMCL, to identify the sub-groups within large clusters such as C1 and C2 when users need to identify more distinct and functionally informative sub-clusters. To increase clustering sensitivity, we reduced the cutoff of GO term size to 2000 and increased the granularity to 1.8, and left similarity cutoff unchanged. With these parameters, GOMCL-sub passed 122 GO terms from cluster C1 and was able to separate them into 4 sub-groups (Figure 3.5A). 117 out of the 122 GO terms were assigned to the two largest sub-groups (C1-1 and C1-2), whose main functional themes were associated with development processes and cellular component organization, respectively (Supplement file 3.4). These two sub-groups recapitulated and extended the main theme of the original cluster, C1. Additionally, this led to the identification of more informative details of the cluster. For instance, the development
process associated with cluster C1-1 was mainly composed of GO terms involved in anatomical

development processes (e.g. root development) (Figure 3.5B, Supplement file 3.5). Whereas, over 85% of the GO terms in cluster C1-2 were involved in processes such as cellular component organization that represented chromosome organization and cytoskeleton organization. More importantly, cell cycle processes were found to intersect with cellular component organization and seemed to link different processes assigned to the C1-2 sub cluster (Figure 3.5C, Supplement
file 3.5). These results combined, provided a more detailed overview of how development processes and cell cycle regulation were different between the two cell populations in the target study. Contrasting to cluster C1, cluster C2 did not appear to contain additional sub-cluster structure that could be separated further, when GOMCL-sub was applied. The visualization of GO hierarchy of GOMCL clusters and further identification of sub-groups with more specific functional themes by GOMCL-sub greatly contribute to biological interpretations by facilitating objective clustering and extraction of overrepresented functional associations. Notably, GOMCL identified these 5 clusters out of a list of 251 GO terms and generated associated result files in ~2 minutes while using only 300 Mb of RAM (easily found in most desktops/laptops). GOMCL-sub further separated cluster C1 and C2 clusters in less than 2 mins with similar amount of memory used. Given the efficiency of the toolkit, this can be easily implemented to conduct batch processing of multiple datasets associated with large –omics datasets.

While the proof of concept analyses described above using GOMCL is used to highlight functional associations drawn from a typical RNAseq experiment, the use of GOMCL is not limited to summarizing functional processes from RNAseq data. For example, it has been recently successfully used in summarizing gene functions associated with multiple epigenetic marks in rice under phosphorus starved conditions (Foroozani et al., 2020). Additionally, GOMCL can be used to cluster and summarize biological processes associated with GO-slim ontologies (Gene Ontology Consortium, 2004), similar to its use with the standard GO terms. To further assess and highlight its versatility, we compared the features of GOMCL to several
existing GO term clustering tools in Table 1.

Table 3.1. Feature comparison of GOMCL and other tools for clustering of GO terms.

<table>
<thead>
<tr>
<th>Feature</th>
<th>GOMCL</th>
<th>Enrichment Map (Merico et al., 2010)</th>
<th>ClueGO (Bindea et al., 2009)</th>
<th>DAVID (Huang et al., 2009)</th>
<th>POSOC (Joslyn et al., 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clustering</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Clustering basis</td>
<td>Jaccard Coefficient/Overlap Coefficient</td>
<td>Jaccard Coefficient/Overlap Coefficient</td>
<td>Kappa statistics</td>
<td>Kappa statistics</td>
<td>GO hierarchical distance</td>
</tr>
<tr>
<td>Clustering method</td>
<td>Markov Clustering (MCL) algorithm</td>
<td>Visual identification</td>
<td>Iterative merging</td>
<td>Iterative merging</td>
<td>Ranking</td>
</tr>
<tr>
<td>Sub-clustering</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Type of visualization support</td>
<td>Network and Hierarchy</td>
<td>Network</td>
<td>Network</td>
<td>Table</td>
<td>Table</td>
</tr>
<tr>
<td>Compatible with other enrichment tools</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Batch processing</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

**Discussion**

GOMCL is an open-source Python toolkit to identify clusters among GO term similarity networks using the MCL clustering algorithm. This toolkit allows grouping of GO terms into functional clusters to further simplify the interpretation of large datasets, reduce redundancy of functional interpretations, and especially when visual identification of cluster structure is not feasible due to a large number of enriched GO terms often found in -omics data (see Table 1 for a comparison with other available tools). To better evaluate and understand the resulting clusters, GOMCL offers options to visualize similarity indexes between GO terms and GO hierarchy. A second module, GOMCL-sub, is further introduced to further examine large clusters when users suspect that two or more distinct minimally overlapping functions might be captured in one large cluster. We demonstrated the use of GOMCL in successfully capturing the functional themes.
associated with a published study (Wendrich et al., 2017) as proof of concept of the toolkit. We showed that GOMCL built a concise and informative view of biological processes different between the two conditions tested in the target study and summarized the main differences. It was also demonstrated that sub-clustering enabled by GOMCL-sub was able to provide additional insight of selected clusters produced by GOMCL to guide further investigation.

GOMCL can be used for batch processing of multiple enrichment test results defined by the user. It is applicable for any research project where lists of genes of interest are generated. It is compatible with a wide variety of GO enrichment analysis tools publicly available, which would reduce intermediate steps needed to convert different input formats to conform to GOMCL requirements.

GOMCL currently uses the MCL algorithm for cluster identification and is compatible with commonly-used GO enrichment tools. For future versions, we consider implementing additional clustering methods, improving labeling of nodes, and supporting more GO enrichment analysis tools.

**Conclusion**

Lists of overrepresented GO functions from GO enrichment analyses are often long and redundant. We present GOMCL as a convenient toolkit to identify functional clusters among GO term similarity networks and further separate the resulting clusters into more informative sub-groups. It enables the user to effectively summarize long lists of GO functions into biologically informative non-redundant clusters without hand-picked selections and look for major functional themes associated with the experiments. GOMCL assists with the unmet yet increasing need for interpreting large gene sets often produced from -omics studies.
CHAPTER 4: CROSS SPECIES MULTI-OMICS REVEALS CELL WALL SEQUESTRATION AND ELEVATED GLOBAL TRANSCRIPTION AS MECHANISMS OF BORON TOLERANCE IN PLANTS*

Introduction

In 1899 the eminent botanist Edwin Copeland stated that “boron produced monstrosity” to describe plant damage due to excess boron (Copeland and Kahlenberg, 1899). Boron functions as an essential micronutrient in plants at a narrow concentration range (0.5 - 1 ppm, equivalent to 46.2 - 92.5 µM in hydroponic media), causing severe growth defects in many plants, including most crops, at only slightly higher concentrations (Brenchley, 1914; Haas, 1929; Warington, 1937; Eaton, 1940; Goldberg, 1997; Reid, 2007b, 2013; Julkowska, 2018; Landi et al., 2019). Early surveys of boron toxicity effects led plants to be classified as sensitive, semi-tolerant, or tolerant to boron (Eaton, 1935). Subsequently, the Food and Agriculture Organization of the United Nations recommended a soil boron level of less than 1.4 mM for even the most tolerant crops to minimize losses in productivity (Eaton, 1944; Ayers and Westcot, 1985; Grieve et al., 2011). The negative impact of boron toxicity on US agriculture was recognized early on (Cook and Wilson, 1918; Eaton, 1935). It is also known to reduce crop yields on all continents where agricultural regions are affected by naturally high amounts of boron in soils or in irrigation water, particularly when the water is obtained from sources near active geothermal areas (Nable et al., 1997; Camacho-cristóbal et al., 2008; Reid and Fitzpatrick, 2009b). Moreover, most soils containing toxic levels of boron occur in semi-arid environments where drought and high salinity compound the stresses on the crops (Reid, 2010).

Excess boron inhibits plant growth by decreasing chlorophyll content, stomatal conductance, photosynthesis, and leads to premature death of shoots and roots (Lovatt and Bates, 1984; Reid et al., 2004; Miwa et al., 2007). Nevertheless, the molecular targets of excess boron and the cellular and molecular processes interrupted by boron stress are poorly understood. Similarly, we have little understanding of the genetic mechanisms underlying boron toxicity responses or the adaptive mechanisms plants use to counter excess boron (Reid et al., 2004; Ruiz et al., 2003; Princi et al., 2016).

In this study, we used the boron-tolerant extremophyte Schrenkiella parvula (formerly Thellungiella parvula and Eutrema parvulum, family Brassicaceae) (Dassanayake et al., 2011; Zhu, 2015; Kazachkova et al., 2018) and its close relative A. thaliana, a boron-sensitive model, to identify cellular processes interrupted by excess boron and to determine the transcriptional and metabolic processes that support growth during boron toxicity. S. parvula is adapted to high levels of boron naturally present in its native habitats in the Central Anatolian plateau of Turkey (Helvaci et al., 2004). The ecotype (Lake Tuz) used in our study was collected from the Lake Tuz region of Turkey and experiences an average concentration of boron (2.2 mM) that is highly toxic to most plants (Nilhan et al., 2008). It can survive soil boron levels as high as 5.8 mM boron in the wild (Nilhan et al., 2008) and 10 mM boron given for two weeks in controlled environments (Oh et al., 2014b). However, the mechanisms that allow S. parvula to grow in the presence of boron concentrations that are toxic to A. thaliana are not known.

Here, we used comparative genomics, transcriptomics, ionomics, and metabolomics to study boron toxicity responses and tolerance in A. thaliana and S. parvula. Our data suggest that excess boron disturbs cell wall metabolism and RNA metabolism-related processes, particularly translation. The cell walls of A. thaliana and S. parvula serve as a sink to partially sequester
excess boron under high boron conditions. *S. parvula* accumulated less boron than *A. thaliana* under boron toxicity, likely through an efficient efflux system. We propose that *S. parvula* has a pre-adapted transcriptome to facilitate rapid metabolic changes when exposed to excess boron and that such a pre-adaptation distinguishes boron stress-adapted and -sensitive plants. We provide a model depicting critical cellular processes that are affected by excess boron and the molecular mechanisms boron stress-tolerant plants use to minimize the growth inhibitory effects of this element.

**Materials and Methods**

**Plant material and growth conditions**

*Schrenkiella parvula* (ecotype Lake Tuz) and *Arabidopsis thaliana* (ecotype Col-0) seeds were surface sterilized with Clorox diluted 1:1 containing 0.05% Tween-20 and 70% ethanol, followed by 4-5 washes with sterile dH₂O. Sterilized seeds were stratified for 4 days at 4 °C in the dark.

Plants for RNAseq, metabolomics, and ionomics experiments, were grown hydroponically in 1/5-strength Hoagland’s solution (Liu et al., 2010; Wang et al., 2018) at 22°C to 24°C in a growth chamber with a 14-h-light/10-h-dark cycle; 100-150 µmol m⁻² s⁻¹ light intensity. 4-week-old plants were transferred to growth media containing fresh 1/5-strength Hoagland’s solution or Hoagland’s solutions containing 5, 10, or 15 mM boric acid. The pH of the media was measured and adjusted to match control solutions in growth media allocated for boric acid treatments. These were kept in the same growth chambers until sample harvest.

For seedlings grown on plates, sterilized seeds were germinated on 1/4-strength Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962). 8-day-old seedlings were transferred to
1/4-strength MS medium with different concentrations of boric acid as indicated in Figure 4.1 and grown in the same growth chamber as described for 4-week old plants.

**Measurement of chlorophyll and root length**

Chlorophyll concentrations were determined on a fresh-weight basis. Leaves of 4-week-old *S. parvula* and *A. thaliana* plants were harvested and weighed. Total chlorophyll was extracted with dimethyl sulfoxide solvent (VWR, Radnor, PA), and measured using a SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA) as described (Richardson et al., 2002). Four biological replicates were used for control and treatments.

Root length was measured daily for 7 days for seedlings grown vertically on 1/4-strength MS agar plates. Root length was measured by marking root tip positions daily at the same time for 7 days for both species. On day 7, the plates were scanned and the root lengths quantified using ImageJ (Schneider et al., 2012). Four biological replicates were used with at least 8 seedlings per replicate.

**Elemental analysis**

Shoot and root tissues were harvested at 3 or 24 hours following control (mock) and 5 and 10 mM boric acid treatments. Samples were dried at 37°C for one week in a desiccator to yield between 5 and 60 mg of dry tissue. For quantification of cell wall elements, we prepared cell walls as an alcohol insoluble residue (AIR) as described (Pettolino et al., 2012). Briefly, the harvested tissues were ground to a powder and washed with aq. 80% ethanol, acetone, and methanol. Selected elements (Li, B, Na, Mg, Al, P, S, K, Ca, Fe, Mn, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, and Cd) were quantified using inductively coupled plasma mass spectrometry (ICP-MS) at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS)-Plant Genetics Facility at the Donald Danforth Plant Science Center as described (Baxter et al.,
Four to five biological replicates were used for each data point. All measurements were normalized to amount per unit weight. One-way ANOVA followed by Tukey’s post-hoc tests implemented in R were used to identify significant differences between samples.

A second independent ICP-MS analysis with a modified protocol that included a rigorous digestion step was conducted to quantify the boron content in the cell walls and to confirm results obtained in the first ICP-MS quantification. AIR from a second set of four biological replicates were prepared as described below. The AIR was digested with 1 mL ultrapure 70% nitric acid (BDH Aristar® Ultra, VWR, Radnor, PA) in 15 mL Teflon beakers, followed by a serial digestion with 100 μL 70% nitric acid on a 100 °C hot plate overnight. Digestions were carefully dried down to almost complete dryness between each step. Acid washed teflon beakers and trace metal clean tubes were used instead of standard laboratory glassware that contain borosilicate in order to minimize the boron background. A final digestion was performed with 100 μL 70% nitric acid and 50 μl 35% H2O2 (ACS grade, Ward’s Science, Rochester, NY) since undissolved particles remained in solution at the end of the second digestion. The samples were dried on a hot plate as described earlier and the residue dissolved in 5 mL 2% nitric acid. The solution was sonicated for 3-5 mins using an ultrasonic cleaner (FS220, Thermo Fisher Scientific, Waltham, MA). The solution was diluted to 10 mL with 2% nitric acid. Boron was quantified using a Thermo iCap Qc ICP - MS (Thermo Fisher Scientific Inc., Waltham, MA). Internal standard solutions containing 6Li, 45Sc, 89Y, 103Rh, 115In, 193Ir, 209Bi were added prior to analysis via a Y-split. Quantification was performed using commercially available standards (IV-ICPMS-71A, Inorganic Ventures, Christiansburg, VA). Pearson correlation coefficient between the two independent ICP-MS experiments was computed using the cor.test function in R.
Identification of orthologs between *S. parvula* and *A. thaliana*

Genome annotations for *S. parvula* version 2.2 (https://phytozome-next.jgi.doe.gov/) and *A. thaliana* genome version 10 (https://www.araport.org/) were used for ortholog identification. When multiple spliced forms existed in *A. thaliana*, the longest version was considered. Orthologous gene pairs as best reciprocal hits between these two species were identified using the CLfinder-OrthNet pipeline with default settings (Oh and Dassanayake, 2019). To account for lineage-specific gene duplications in both species, orthologous gene pairs were searched reciprocally between the two species using BlastP with an e-value of 1e-5 and MMseqs2 (Steinegger and Söding, 2017) with an equivalent e-value cutoff. These pairs were further filtered using OrthoFinder (Emms and Kelly, 2015) with granularity -I of 1.6 and were added back to the CLfinder pipeline to extract all possible ortholog pairs between the two species. Among a total of 27,206 *A. thaliana* protein-coding gene models, 22,112 were paired with at least one *S. parvula* homolog. Similarly, 21,673 out of 26,847 *S. parvula* gene models were paired with at least one *A. thaliana* ortholog. The two reciprocal searches were merged, and redundant pairs were removed to generate 23,281 *S. parvula*-*A. thaliana* orthologous gene pairs.

Transcriptome profiling

Root and shoot tissues were harvested separately for each plant 24 hours after boric acid treatment. Total RNA (at least 6 µg) was extracted using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), with an additional step to remove contaminating DNA. Four biological replicates per condition were generated and three were used for RNA-seq libraries. RNA-seq libraries were prepared with a TruSeq Stranded mRNAseq Sample Prep kit (Illumina, San Diego, CA, USA) at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Libraries were barcoded and sequenced on three lanes of HiSeq2500 platform
(Illumina), generating > 25 million high-quality 100-nucleotide (nt) single-end RNA-seq reads per sample. These reads are deposited in the BioProject PRJNA663969 at the NCBI-SRA database.

RNA-seq reads after quality checks using FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) from each sample were mapped to either A. thaliana TAIR10 or S. parvula genome v2 using HISAT2 version2.0.1(Kim et al., 2015) with default parameters. A custom Python script was used to count uniquely mapped reads to each gene model found to be expressed. To identify a list of robust differentially expressed genes (DEGs), we used a consensus list from DEGs identified using a parametric method, DESeq2 (Love et al., 2014) and a non-parametric method, NOISeq (Tarazona et al., 2015) with a FDR-adjusted p-value cutoff set to 0.05. Only genes selected by both methods as significantly different were used for downstream analyses.

To compare the expression levels of orthologs between S. parvula and A. thaliana, expression values of reads per kilobase of transcript per million mapped reads (RPKM) < 1 were removed. The RPKM values of filtered ortholog pairs were converted to log2-transformed counts and median-normalized. These normalized RPKM values for ortholog pairs across all samples from shoots and roots were subjected to fuzzy k-means clustering (Gasch and Eisen, 2002) to identify co-expressed gene groups. Ortholog pairs in each of the resulting clusters were further filtered based on: (1) the membership of a given ortholog pair was no less than 0.5; (2) the expression changes of each ortholog pair in a given cluster were considered to be statistically significant by both DESeq2 and NOISeq to ensure that the expression pattern of a given pair agreed with that
for the cluster; and (3) clusters in which the pattern was consistent between all biological replicates were considered for downstream analyses.

BiNGO (Maere et al., 2005) was used to identify enriched networks of Gene Ontology (GO) terms in each species. To reduce the redundancy between enriched GO terms and their associated inference related to DEGs, redundant GO terms with > 50% overlap with similar terms were further clustered using Markov clustering implemented via GOMCL (https://github.com/Guannan-Wang/GOMCL) (Wang et al., 2020b). Custom Python scripts were used to extract all direct child terms of a given GO term or all genes annotated with the given GO term. GO terms with zero assigned genes from *A. thaliana* were removed from the analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2016) was used to map genes to specific metabolic pathways.

**RT-qPCR**

Plants were grown and treated with excess boric acid and harvested as described for RNA-seq experiments. Total RNA (0.5 µg) was used in a 20 µL reverse transcription (RT) reaction for first-strand cDNA synthesis with SuperScript™ III Reverse Transcriptase according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The reverse transcription products were diluted to 200 µL, and 2 µL was used in a 20 µL qPCR reaction using SYBR™ Select Master Mix (Applied Biosystems, Foster City, CA) in a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Our transcriptomic profiles showed that the commonly used reference genes, *ACT2*, *CYTC-1*, *CYTC-2*, *EF1α*, *UBQ10*, and *GAPDH*, all had expression levels that varied between treatments, plants, and tissue types, which made them unsuitable as reference genes in RT-qPCR. Therefore, we searched for the most uniformly expressed and conserved genes in both plants and selected
At5g46630 (ADAPTOR PROTEIN-2 MU-ADAPTIN) and At4g26410 (RGS1-HXK1 INTERACTING PROTEIN 1) and their S. parvula orthologs as internal reference genes, following the best practice recommendations from previous studies (Czechowski et al., 2005; Wang et al., 2014).

**Metabolomics analyses**

Shoot and root samples were harvested at 24 hours after control, 5, and 10 mM boric acid treatments and freeze dried (FreeZone 2.5 Plus, Labconco Corp., Kansas City, MS). Untargeted profiling of polar metabolites, including boric acid, using gas chromatography-mass spectrometry (GC-MS) was performed at the Metabolomics Center at University of Missouri, Columbia. To facilitate the detection of trace metabolites, 20 mg of roots or 50 mg of shoots from pools of 7-12 plants were used per biological replicate per condition. The dry tissues were suspended in 1.0 ml of aq. 80% methanol and 20 µl of HPLC grade water containing 1 µg/ml ribitol. The suspensions were vortexed for 20 seconds, and sonicated for 15 min. The suspensions were shaken for 2 hours at 140 rpm in an orbital shaker and centrifuged for 30 minutes at 15000 g. Equal amounts of the supernatant were transferred to autosampler vials. The solutions were concentrated to dryness using a gaseous nitrogen stream. The dried extracts were methoximated with 25 µl of 15 mg/mL methoxyamine hydrochloride in pyridine, and trimethylsilylated with 25µL N-methyl-N-(trimethyl-silyl)trifluoroacetamide (MSTFA) and 1% chlorotrimethylsilane (TMCS). The derivatized extracts were analyzed for non-targeted metabolic profiling using an Agilent 6890 GC coupled to a 5973N MSD mass spectrometer with a scan range from m/z 50 to 650 (Agilent Technologies, Inc., Santa Clara, CA). 1 µl of sample was injected into the GC column with a split ratio of 1:1 for polar GC-MS analysis. Separation was achieved using a 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 um film
thickness) with a temperature program of 80 °C for 2 min, then ramped at 5 °C /min to 315 °C and held at 315 °C for 12 min, and a constant flow of helium gas (1.0 ml/min). A standard alkane mix was used for GC-MS quality control and retention index calculations. The raw data were first deconvoluted using AMDIS software (http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis) and annotated through mass spectral and retention index matching to an in-house spectra library. The unidentified compounds were searched and identified using spectral matching to a commercial NIST17 mass spectral library. The raw abundance/intensity for each identified compound was normalized with the internal standard, ribitol (peak area of each metabolite/peak area of internal standard × 1,000).

Different molecular features were manually curated to the most relevant molecular feature for each identified metabolite. A minimum of three biological replicates were used for each condition for each tissue. Significant differences between samples were determined by Student’s t tests followed by FDR correction for multiple testing (Benjamini and Hochberg, 1995) in MetaboAnalystR (Chong et al., 2019).

**Yeast complementation assay**

Yeast Δbor mutant strains were in the MATa ADE2 his3Δ1 leu2Δ0 lys2Δ0 TRP1 ura3Δ0 bor1D::KanMX background. The entire coding regions of AtBOR4, AtBOR5, SpBOR4, SpBOR5 were cloned from A. thaliana and S. parvula cDNA, respectively, and were separately introduced into the pDD506 plasmid (Wang and Donze, 2016), driven by the ADH1 promoter. The Δbor mutant was transformed with the recombinant pDD506 plasmids or the empty pDD506 plasmid as a negative control. The transformants were selected on SD medium-His. Boron toxicity tolerance assays were performed as described in Nozawa et al. (2006). Briefly, yeast cells were grown in SD medium to OD$_{600}=1$, collected, and spotted onto solid SD or SD containing 80 mM
boric acid with different titers. The plates were photographed after incubation for 10 days at 30 °C.

Results

*S. parvula* accumulates less boron while sustaining growth for longer durations compared to *A. thaliana*

*S. parvula* was unaffected by treatments of 5, 10, or 15 mM boric acid whether grown

Figure 4.1. *A. thaliana* and *S. parvula* respond to boric acid treatments differently. (A) Hydroponically grown 33-day-old *A. thaliana* and *S. parvula* with different concentrations of boric acid. Boric acid treatments started at 4-week-old plants. Scale bars = 5 cm. (B) Growth phenotype of 2-week-old *A. thaliana* and *S. parvula* on plates with boric acid. Plants were germinated and grown on 1/4 MS medium, transferred to 1/4 MS medium supplemented with boric acid one week after the germination. (C) Dry biomass and (D) total chlorophyll content of hydroponically grown *A. thaliana* and *S. parvula*. (E) Root growth, (F) lateral root density, and (G) average lateral root length of plate-grown *A. thaliana* and *S. parvula* seedlings. (H) Boron and (I) free boric acid accumulation in shoots and roots in *A. thaliana* and *S. parvula*. In panels C-I, all values are mean ± SD (n=3~5, except for E where n=14~15). Asterisks represent significant differences (p<0.05) compared to control determined by either one-way ANOVA followed by Tukey’s post-hoc tests (C-H) or Student's t-test (I).
hydroponically for four weeks (Figure 4.1A) or on plates for one week (Figure 4.1B). In contrast, *A. thaliana* showed clear growth inhibition, wilting, and chlorosis of leaves 5 or 7 days after the boric acid treatments (Figure 4.1A and B). The control growth media included ~100 μM boron to provide a boron-sufficient growth medium, and the treatments added excess boron to this growth-sufficient level. We observed a substantial reduction in the fresh and dry weights of *A. thaliana* shoots and roots in response to excess boron, whereas *S. parvula* biomass was unaffected by the treatments (Figure 4.1C). Similarly, total chlorophyll content decreased in *A. thaliana* but remained unchanged in *S. parvula* at 3 days after the treatments (Figure 4.1D). In *A. thaliana*, we observed dose-dependent inhibitory effects of excess boron on root growth, whereas *S. parvula* root growth was not affected (Figure 4.1E). In *A. thaliana*, the 15 mM boron treatment led to a significant reduction of lateral root density (Figure 4.1F), while average lateral root length was decreased in all treatments (Figure 4.1G). In contrast, neither lateral root density nor average lateral root length of *S. parvula* was affected by excess boron (Figure 4.1F and G).

We quantified the concentration of boron (on a dry weight basis) in shoots and roots of plants exposed to excess boron using inductively coupled plasma mass spectrometry (ICP-MS) to determine if the distinct boron stress-responses between *S. parvula* and *A. thaliana* reflected differences in their boron accumulation (Figure 4.1H). The initial boron levels in roots and shoots of control plants were similar for both species. Differences in boron accumulation only differed in response to the excess boron treatments. The levels of boron increased significantly in the roots and shoots of both species over time and with higher concentrations of boron. However, the level of boron that accumulated in *S. parvula* was lower than in *A. thaliana*. This was most apparent in roots, 24 hours after the 5 mM boric acid treatment, in which boron levels increased 14-fold in *A. thaliana* but were unchanged in *S. parvula* (Figure 4.1H). The greater capacity of *S.
parvula to maintain lower boron levels relative to A. thaliana may have contributed to the continued root growth in S. parvula under conditions that decreased A. thaliana root growth (Figure 4.1E). However, S. parvula roots could not limit boron accumulation comparable to control plants when grown on 10 mM boric acid. Nevertheless, under this treatment, the relative accumulation of boron in roots was still much lower in S. parvula than in A. thaliana (Figure 4.1H). In contrast to roots, S. parvula shoots significantly accumulated boron even within 3 hours of the 5 mM boric acid treatment, although the amounts were lower than in A. thaliana shoots under all comparable treatments (Figure 4.1H). This indicated that the overall response to excess boron is different between roots and shoots.

We next determined if the physiological responses (Figure 4.1 A-G) to excess boron coincided with a disruption in the ionic balance of other elements. Reduction of nutrient uptake or concurrent over-accumulation of other elements may cause toxicity symptoms not directly

![Figure 4.2. Ionomic profiles in response to boric acid treatments. Significant differences of each treatment compared to control were based on one-way ANOVA followed by Tukey’s post-hoc tests (p < 0.05).](image-url)
attributed to boron stress. To this end, we quantified 20 other elements known for their presence in plants (Figure 4.2). Neither species showed dramatic changes to their ionomic profiles during boron treatments except for the expected increase in boron content, suggesting that the observed physiological responses in both species were largely caused by the cellular disturbances due to excess boron accumulation.

**High external boron results in a substantial increase in free boric acid in A. thaliana roots but not in S. parvula roots**

After being transported primarily as free boric acid into the cytoplasm, boron is either found as free boric acid/borate or bound to organic metabolites within plant cells (Woods, 1996; Hu and Brown, 1997; Brown et al., 2002; Broadley et al., 2012; Camacho-cristóbal et al., 2008). We therefore measured the relative abundance of free boric acid in plants using gas chromatography-mass spectrometry (GC-MS), to assess how *S. parvula* may retain externally supplied boric acid differently from *A. thaliana* (Figure 4.1I). Notably, we detected substantial increases (up to 14 fold) in free boric acid in treated *A. thaliana* shoots compared to the control, which was comparable to the increase in total boron accumulation (Figure 4.1H). In contrast, the increase in free boric acid (up to 4.5 fold, Figure 4.1I) was much lower than the total boron accumulation in *A. thaliana* roots (Figure 4.1H). This suggested that a major fraction of the increased total boron in *A. thaliana* roots was in the form of B-complexes. *S. parvula* shoots were similar to *A. thaliana* roots since the increase in free boric acid (up to 2.2 fold; Figure 4.1I) was much lower than the increase of total boron in treated *S. parvula* shoots (Figure 4.1H). It is equally noteworthy that the levels of free boric acid remained unchanged in *S. parvula* roots under all conditions tested (Figure 4.1I) despite the substantial increases of total boron in plants grown on 10 mM boric acid (Figure 4.1H). This led us to hypothesize that *S. parvula* stores much of the
excess boron in the form of B-complexes and thereby minimizes the accumulation of free boric acid, particularly in roots, more effectively than A. thaliana.

The transcriptomic response to excess boron is greater in A. thaliana than S. parvula

We expected that A. thaliana and S. parvula, which have rapid yet divergent responses to excess boron, would exhibit regulation at the transcriptional level that determined their subsequent responses to this stress. To develop a comparative framework to contrast transcriptional responses to excess boron, plants were transferred to media containing 5 mM boric acid. This concentration was sufficient to induce discernible changes in both species (Figure 4.1), but did

Figure 4.3. Transcriptional responses of A. thaliana and S. parvula to excess boron. (A) Principal component analysis (PCA) differentiates the transcriptomes of control and treated samples from shoot and root tissues of A. thaliana (left) and S. parvula (right). (B) PCA of A. thaliana and S. parvula transcriptomes within shoot (left) and root (right). (C) Expression levels of BOR1, NIP5;1, BOR4, BOR5, BOR7 in control and 5 mM boric acid treatment. Asterisks represent significant differences in expression compared to control (at FDR-adjusted p<0.05) determined by both DESeq2 and NOISeq. (D) Comparison of the basal expression levels of ortholog pairs in roots between A. thaliana and S. parvula. Ortholog pairs that encode boron transporters and channels are marked in red. Gray diagonal dashed line marks identical basal level expression between the two species while ortholog pairs above the red dashed line show >2000-times higher basal expression in S. parvula than in A. thaliana. (E). Growth of yeast Δbor mutants transformed with either ScBOR1, AtBOR4, AtBOR5, SpBOR4, or SpBOR5 on medium containing 0 and 80 mM boric acid. Negative control was transformed with the empty vector.
not cause tissue death in the sensitive model even at 5 days post treatment (Figure 4.1A). We chose a 24-hour duration to allow us to assess transcriptomic responses when neither species showed observable changes to suggest cell death. Since the root stress response was different from that of shoots, we investigated root and shoot transcriptomes separately in our comparative –omics framework.

The largest observed variance (>40%) in the transcriptomes within a species was attributed to the tissue differences (i.e. root versus shoot) as seen in the principal component analysis (PCA) (Figure 4.3A). However, when we compared the transcriptomes of the same tissues across species, the treated \textit{A. thaliana} transcriptomes were strikingly different from the control, whereas \textit{S. parvula} treated and control transcriptomes were almost indistinguishable (Figure 4.3B). This suggested that the extent of the transcriptional adjustment to excess boron is much greater in \textit{A. thaliana} than in \textit{S. parvula}. Indeed, we found 9,657 genes in shoots and 6,126 genes in roots.

![Figure 4.4](image.png)

Figure 4.4. Differential expression visualized using MA-plots from shoot (left panel) and root (right panel) of \textit{A. thaliana} (upper panel) and \textit{S. parvula} (lower panel). Red dots represent up-regulated DEGs and blue dots indicate down-regulated DEGs.
differentially expressed in response to excess boron in *A. thaliana* (Figure 4.4). In contrast, the number of boron stress-responsive genes in *S. parvula* was much smaller (535 in shoots and 63 in roots) (Figure 4.4). The magnitude of the differences in the overall transcriptomes reflected the visible physiological responses of these two species to excess boron (Figure 4.1).

To independently assess the reproducibility of the transcriptomic responses captured by RNAseq, we selected five to six differentially expressed genes per tissue in both species and additional biological replicates to obtain the relative expression of 20 genes using RT-qPCR. We found high concordance in transcript level changes between the RNAseq and RT-qPCR data (Pearson $R^2 = 0.71$, $p = 2.65e-07$) (Figure 4.5).

![Figure 4.5. Assessment of qPCR and RNA-seq expression data agreement for selected differentially expressed genes.](image)

*A. thaliana* and *S. parvula* exhibit differences in boron transporter gene expression and function

Boron uptake and translocation are known to involve a family of boron transporters (BORs) and membrane intrinsic proteins (MIPs) (Diehn et al., 2019; Yoshinari and Takano, 2017). We compared the expression changes of all known boron transporters and channels to determine the
transcriptional responses to excess boron related to boron acquisition and transport (Figure 4.3C, D). *S. parvula* roots showed down-regulation of both *NIP5;1* and *BOR1*, the two dominant transporters mediating boron uptake and xylem loading during boron-deficient conditions (Takano et al., 2002, 2006). *A. thaliana* roots also showed down-regulation of *NIP5;1* (Figure 4.3C). This observation supports the idea that both species attempted to reduce boron uptake as well as boron transport to the shoot in response to excess boron.

Plants may alleviate boron toxicity by activating transporters that export boron back to soil or to other compartments away from actively growing tissues, in addition to minimizing boron uptake. *BOR4* is the only boron transporter demonstrated to alleviate boron toxicity by moving excess boron from roots back to soil (Miwa et al., 2014, 2007). Our transcriptomic data showed that *BOR4* transcript abundance was not affected by excess boron in either *A. thaliana* or *S. parvula* (Figure 4.3C). However, basal transcript levels of *SpBOR4* were ~5 fold higher (22.2 RPKM) than those of *AtBOR4* (3.8 RPKM) in roots (Figure 4.3C, D). We detected two under-studied putative boron transporters (*BOR5* and *BOR7*) that were significantly induced by excess boron. One of these, *BOR5* is the closest homolog of *BOR4* (Takano et al., 2008; Sun et al., 2012; Oh and Dassanayake, 2019). Whereas *BOR5* was induced (~3 fold) by excess boron in *A. thaliana* roots (Figure 4.3C), the *S. parvula* ortholog showed a dramatically higher constitutive expression prior to the stress and represents one of the largest basal expression differences in roots (>2000 fold higher in *S. parvula*) observed among all ortholog pairs between the two species (Figure 4.3D). *BOR7*, encoding another BOR4-like boron transporter (Luo et al., 2019), was also induced in *A. thaliana* roots in response to excess boron, suggesting a putative function to exclude boron under excess boron conditions (Figure 4.3C). However, *BOR7* was hardly detected at basal levels (lower than 0.1 RPKM) in *A. thaliana* or *S. parvula* roots (Figure 4.3C).
Taken together, our data suggest that, under toxic levels of boron, *A. thaliana* induced the transcript levels for boron transporters implicated in boron exclusion from the roots. On the other hand, in *S. parvula* roots, both *SpBOR4* and *SpBOR5*, although not induced by excess boron, were expressed at a basal level much higher than their orthologs in *A. thaliana*. Therefore, we hypothesized that BOR5 is functionally active in excluding boron in *S. parvula* roots exposed to excess boron.

To further assess the role of SpBOR5 as a key contributor for boron exclusion in *S. parvula*, we individually expressed *BOR4* and *BOR5* from each species in a yeast mutant lacking the native boron transporter *ScBOR1* (Figure 4.3E). This Δbor yeast mutant is sensitive to high concentrations of boric acid because of its inability to export excess boron. SpBOR5 fully rescued the growth of Δbor yeast exposed to a toxic level of boron, while *AtBOR4*, *AtBOR5*, or *SpBOR4* failed to complement the Δbor mutant growth defects (Figure 4.3E). This demonstrates that *SpBOR5* functions similar to *ScBOR1* and seems to have a higher boron efflux capacity compared to *AtBOR4*, *AtBOR5*, and *SpBOR4*. These results, together with the strikingly higher basal expression of *SpBOR5* in *S. parvula* roots, suggest that SpBOR5 likely enabled *S. parvula* to exclude excess boron more efficiently than its stress-sensitive relative *A. thaliana*.

**Transcriptomic responses to boron predict altered cell wall metabolism as a major cellular response to enable cell walls to capture excess boron**

We next examined biological processes and functions enriched among the differentially expressed genes (DEGs) in *A. thaliana* roots as it exhibits readily discernible responses when treated with excess boron. From a total of 3,504 boron stress-repressed DEGs, 2,728 could be associated with specific GO functions. Among them, we identified 19 functional clusters using GOMCL (Wang et al., 2020b). In short, GOMCL clustered enriched GO terms that had shared genes (>50%) using Markov Clustering and identified non-redundant representative functions...
within a GO network. The top ten root clusters included over 96% of the GO-annotated DEGs (Figure 4.6A). This approach revealed that cell wall-related processes account for the largest proportion among boron stress-suppressed DEGs in *A. thaliana* roots. Three of the top ten functional clusters (C2, 7, and 8) were associated with cell wall-related processes, accounting for 1,453 DEGs (Figure 4.6A, red boxes).

![Figure 4.6](image)

Figure 4.6. Cell wall metabolism is altered under boron toxicity in *A. thaliana*. (A) Functional clusters enriched among boron stress-repressed genes in *A. thaliana* roots. Clusters associated with cell wall metabolism are marked by red-dashed boxes. Clusters are differently colored and labelled with the representative functional term. Each node represents a GO term; node size represents genes in the test set assigned to that functional term; GO terms sharing more than 50%
of genes are connected with edges; and shade of each node represents the p-value assigned by the enrichment test (FDR-adjusted p<0.05) with darker shades indicating smaller p-values. (B) Changes in gene expression associated with biosynthesis of cell wall components in response to boric acid treatment in A. thaliana shoots and roots. Genes are represented by square blocks, grouped by families or pathways, with up- and down-regulation marked by red and blue, respectively. UDP, uridine diphosphate; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; UDP-GlcA, UDP-glucuronic acid; UDP-GalA, UDP-galacturonic acid; UDP-Xyl, UDP-xylose; UDP-Ara, UDP-arabinose; UDP-Araf, UDP-arabinofuranose; UDP-Rha, UDP-rhamnose; UDP-Api, UDP-apiose; GDP-Man, GDP-mannose; GDP-Fuc, GDP-fucose; GDP-Gal, GDP-galactose; GAE, UDP-D-glucuronic acid 4-epimerase; UXS, UDP-D-xylose synthase; UXE, UDP-D-xylose 4-epimerase; RGP, reversibly glycosylated protein; AXS, UDP-D-apiose/UDP-D-xylose synthase (also known as UAXS); MIOX, inositol oxygenase; UGD, UDP-D-glucose dehydrogenase; UGP/USP, UDP-glucose pyrophosphorylase/UDP-sugar pyrophosphorylase; RHM/UER, rhamnose synthase gene/nucleotide-rhamnose epimerase-reductase; UGE, UDP-D-glucose 4-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GMD/GER, GDP-4,6-dehydratase/GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase; GME, GDP-D-mannose 3,5-epimerase; EXTs, extensins; EXT GTs, extensin glycosyltransferases; EXPs, expansins; XTHs, xyloglucan endotransglucosylase/hydrolases; RGXT, rhamnogalacturonan xylosyltransferase. (C) Major organic metabolic groups that changed their abundance in response to excess boron stress in A. thaliana shoots. For each category, the number and proportion of metabolites that changed in abundance compared to the total identified are shown. (D-E) Monosaccharide precursors of cell wall polysaccharides (D) and sugar alcohols (E) that changed in abundance in shoots of A. thaliana and S. parvula 24 hours after boric acid treatments. The relative abundance is given compared to the internal standard, ribitol. (F) Boron contents in cell walls extracted from shoots and roots of A. thaliana and S. parvula under different treatments for 24 hours. (G) Cell wall yield of shoots and roots in A. thaliana and S. parvula exposed to different treatments for 24 hours. Cell wall yield was calculated as the percentage of plant biomass on a dry weight basis. In panels D-G, all values are mean ± SD (n=3~5). Asterisks represent significant differences (p<0.05) compared to control determined by Student's t-test.

Next, we expanded our analyses of boron stress-responsive DEGs in functionally enriched clusters to all genes known to be involved in the biosynthesis of major cell wall components. We included both root and shoot tissues to view cell wall biosynthesis-related changes at the whole plant level. Figure 4.6B summarizes the cell wall biosynthesis pathways, including precursors, intermediates, and the building blocks of cell wall components, together with the genes involved in the process. Cellulose, hemicellulose, and pectin constitute about 90% of cell wall mass.
Albersheim et al., 1996; Held et al., 2015). Cellulose is synthesized at the plasma membrane by cellulose synthase (CesA) complexes (Schneider et al., 2016; McFarlane et al., 2014), whereas pectins and hemicelluloses are assembled in the Golgi and then exported to the apoplast (Kousar et al., 2012). Hemicelluloses include xyloglucans, xylans, mannans, glucomannans, and mixed-linkage glucans (MLG) (Scheller and Ulvskov, 2010; Pauly et al., 2013), while the predominant pectins are homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) (Atmodjo et al., 2013).

We found that a total of 14 out of 25 galacturonosyltransferases (GAUTs) and GAUT-like (GATL) genes, which were shown or suggested to be involved in pectin biosynthesis, were DEGs in A. thaliana shoots and roots, of which 13 were induced in shoots (Figure 4.6B). All the glycosyltransferases that have been proven or suggested to code for genes (4 RGXTs and 2 SIAs) involved in assembling the side chains of RG-II on a HG backbone showed a 4-fold or higher increase in A. thaliana shoots (Figure 4.6B). Those glycosyltransferases in the roots, however, did not show this induction (Figure 4.6B). Synergistically, several genes coding for the enzymes (UXSs and UXEs) producing UDP-arabinopyranose (UDP-Arap) and UDP-arabinofuranose (UDP-Araf), which are the donors used by glycosyltransferases to incorporate Arap and Araf into RG-I and RG-II (Bar-Peled and O’Neill, 2011), were also induced in A. thaliana shoots, while the majority of other NDP-sugar biosynthesis genes were repressed by excess boron (Figure 4.6B, left panels). Contrasting to the transcriptomic signal suggesting increased pectin components, the expression of all 10 CesA genes coding for cellulose synthases (Carroll and Specht, 2011; McFarlane et al., 2014) together with other genes mostly associated with hemicellulose biosynthesis remained unaffected (Figure 4.6B). The only experimentally verified molecular function of boron in plants is to cross-link RG-II-pectins in the cell wall (Kobayashi et
Therefore, a net induction of genes associated with pectin biosynthesis in *A. thaliana* shoots suggested a path to potentially bind more boron and trap excess boron in shoot cell walls. The cell wall also contains structural hydroxyproline-rich glycoproteins (HRGPs), notably the extensins (Cannon et al., 2008; Lamport et al., 2011). In *A. thaliana* shoots, excess boron significantly induced genes coding for multiple extensins and glycosyltransferases (GTs) involved in the extensin glycosylation (Velasquez et al., 2011) (Figure 4.6B and Figure 4.7). It is notable that *A. thaliana* root and shoot expression profiles differed substantially. In roots, virtually all the transcripts potentially coding for extensins that significantly responded to excess boron were suppressed whereas in shoots, transcripts coding for extensins and associated GTs were all induced (Figure 4.6B). Such a differential regulation of the extensins suggests that the shoot cell wall may be stiffer in *A. thaliana* under excess boron than in the control (Figure 4.7).

![Figure 4.7. Summary of cell wall modifications in *A. thaliana* in response to boric acid treatment. Genes are represented by in colored blocks, grouped into families and pathways, with up- and down-regulation marked by red and blue, respectively.](image)

Stiffening of cell walls has been reported in plants under other abiotic stresses (Tenhaken, 2015). In line with this finding, we also noted the co-repression of many of the genes encoding catalysts...
of cell wall loosening, including expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) (Cosgrove, 2016) in both shoots and roots (Figure 4.6B, Figure 4.7). The prominent changes in transcriptional responses related to cell wall biology observed in A. thaliana led us to hypothesize that cell walls serves as a sink to store excess boron under boron stress and the associated cell wall modifications were initiated as a transcriptional cascade of several processes including cell wall organization; synthesis and regulation of nucleotide sugar transporters that are linked to cell wall sugars; structural glycoproteins; and cell wall interacting kinases (Figure 4.6 A-B).

**Cell wall pectin precursor abundance significantly increased in response to excess boron in A. thaliana shoots**

Based on the transcriptomic signature that suggested major cell wall modifications, especially related to pectins in A. thaliana shoots, we next assessed if such modifications coincided with changes in metabolic pools in boron-stressed shoots. We used GC-MS metabolic profiling to capture the primary metabolite pools from control and treated tissues of A. thaliana and S. parvula. We detected 167 and 263 annotated metabolites that were differentially accumulated under 5 mM and 10 mM boric acid treatments, respectively. By contrast, the relative abundances of any of these metabolites did not change significantly in S. parvula shoots exposed to excess boron.

We grouped the functionally annotated organic metabolites that significantly changed in their abundance in response to excess boron into five categories. At least one third of these metabolites were sugars or sugar derivatives, including sugar alcohols, while the remaining pool primarily consisted of amino acids and other amines, fatty acids, and other organic acids (Figure 4.6C). The organic acids included pyruvic acid, citric acid, and succinic acid. Their abundances
are known to change in response to abiotic stresses, which is often associated with changes to overall energy balance during stress (Treves et al., 2020).

In line with our transcriptomic data, the relative abundance of many of the free monosaccharides that are components of cell wall polysaccharides, including arabinose, galactose, rhamnose, xylose, and mannose significantly increased upon excess boron in A. thaliana shoots (Figure 4.6D and Figure 4.8). Remarkably, the abundance of none of these sugars significantly changed in boron-stressed S. parvula (Figure 4.6D). Arabinose, which is a component of RG-I and RG-II pectic polysaccharides (Bar-Peled and O’Neill, 2011), increased >2.5-fold, together with xylose, the precursor of arabinose (Atmodjo et al., 2013; Seifert, 2018) in response to excess boron in A. thaliana shoots (Figure 4.6D). Rhamnose, which is present in the side chains of RG-II and the backbone of RG-I (Bar-Peled and O’Neill, 2011), also showed a significant increase in response to 10 mM boric acid in A. thaliana shoots, together with galactose, another component of RG-I and RG-II. Similarly, the precursors of other cell wall polysaccharides, including mannose,
fructose, and glucose increased in response to 10 mM boric acid (Figure 4.6D). Remarkably, none of these sugars changed significantly in S. parvula shoots during any of the boron treatments (Figure 4.6D). The overall changes in free monosaccharides during excess boron treatment of A. thaliana shoots support the view that pectic polysaccharides provide binding sites to trap excess boron in the cell walls.

We found that several sugar alcohols in A. thaliana shoots, including myo-inositol, cellobiotol, galactinol, erythritol, and glycerol, also increased in response to excess boron (Figure 4.6E). Myo-inositol is a precursor of pectin and hemicellulose (Kanter et al., 2005; Endres and Tenhaken, 2009), and its increase is consistent with our working model that cell wall pectins capture excess boron during boron stress. Cellobiotol and galactinol are also presumed to be involved in cell wall carbohydrate metabolism (Unda et al., 2017). Moreover, some of these sugar alcohols could bind excess boron in a manner similar to sorbitol and mannitol (Brown and Hu, 1998b, 1996; Brown et al., 1999). Taken together, our metabolic profiling provide evidence that excess boron led to the increase in sugars and sugar alcohols, many of which are either directly or indirectly related to cell wall polysaccharides in A. thaliana.

**Boron accumulates in the cell walls of A. thaliana and S. parvula**

We next determined if A. thaliana and S. parvula accumulated boron in their cell walls when grown in excess boron using ICP-MS. In A. thaliana, cell wall boron increased in roots and shoots of treated plants compared to the control group (Figure 4.6F). By contrast, we only detected an increase in boron in S. parvula root cell walls (Figure 4.6F). Cell wall yield remained constant under all tested conditions (Figure 4.6G), even 5 days after the treatments (Figure 4.9). We performed a second series of experiments with four additional biological replicates using an extensive digestion procedure while minimizing possible contaminating boron sources during the
experiment (see Methods) to validate boron sequestration in the cell wall during excess boron treatment. These results were consistent with the cell wall boron quantifications we initially performed (Pearson correlation coefficient $r = 0.76$, $P = 0.015$) (Figure 4.10), and confirmed that boron accumulated in the cell walls of *A. thaliana* and *S. parvula*.

We next determined if other elements present in plant tissues accumulated in the cell walls as a result of excess boron treatments. None of the 20 elements analyzed differed significantly from

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Figure 4.9. Cell wall content in *A. thaliana* and *S. parvula* treated with excess boron for 5 days. Values shown are mean ± SD (n = 3 or 4).

Figure 4.10. Conformance of boron content quantified using two independent experiments. Fold changes were calculated comparing the treatment to the control for each tissue from each species. Pearson’s $r$ and $p$-values are indicated.
control plants in cell walls from *S. parvula* roots and shoots and *A. thaliana* shoots (Figure 4.11).

![Figure 4.11. Ionomic profiles in the cell wall extracts in response to boric acid treatments at 24 hours. Significant differences of each treatment compared to control for each element were based on one-way ANOVA followed by Tukey’s post-hoc tests (p < 0.05).](image)

By contrast, almost half of the elements decreased in abundance in root cell walls of excess boron-treated *A. thaliana* (Figure 4.11). This is likely related to the substantial root growth inhibition observed specifically for excess boron-treated *A. thaliana*.

Our results, when taken together, suggest that cell walls do capture excess boron. Additionally, *A. thaliana* shoot cell walls have a higher capacity to retain boron than their root counterparts (Figure 4.6F). Since cell wall yield did not change in response to excess boron, the observed changes are likely due to alterations in the internal structures of the cell walls to enable compartmentalization of excess boron. We also observed that the increase in the boron content in *A. thaliana* shoot and root cell walls (>2 fold) was smaller than the increase in boron content in the entire tissue (compare Figures 1H and 3F). This was most notable in *A. thaliana* roots where whole-tissue boron content increased up to 27 fold. A less pronounced, but similar trend was observed for *S. parvula* roots (Figure 4.6F). Therefore, cell walls may only provide a partial sink for excess boron, and cellular processes involved in cell wall modifications may be limited in the amounts of boron that they can sequester.
Altered RNA metabolism in response to excess boron led to an increased mean expression of the entire transcriptome in *A. thaliana* roots and shoots

We next searched for cellular processes that may serve as additional substrates for excess boron or molecular targets that may cause cellular toxicity if bound by excess boron in the cytoplasm. We expected that the genes induced by excess boron may shed light on such processes. Our analysis identified 19 functional clusters comprised of 2,112 of the 2,622 boron stress-induced genes in *A. thaliana* roots, of which the top ten largest clusters represented 99% (2,094) of the total number of genes from all clusters. The most notable cellular process among the induced genes in roots was RNA metabolism described by the largest functional cluster (C1 in Figure 4.12A). Further, clusters C5, included transcription and translation regulation, and C9, represented by ribosome organization, are also associated with RNA metabolism (Figure 4.12A, red boxes). This indicates that in *A. thaliana* roots, RNA metabolism-related processes were substantially affected by boron toxicity.

To further investigate how RNA metabolism could be altered by excess boron, we examined all genes represented by RNA metabolism (GO:0016070), together with their regulators annotated under specific child GO terms in the *A. thaliana* genome. Interestingly, all RNA metabolism processes, as well as translation and ribosome biogenesis, were enriched in genes differentially responsive to excess boron in our study (Figure 4.12B). This further confirmed our earlier observation of RNA metabolism being a major target of boron stress, especially in *A. thaliana* roots (Figure 4.12A). There were many more boron stress-induced genes than repressed genes in most of the GO categories especially in *A. thaliana* roots (Figure 4.12B). For example, RNA processing, RNA modification, ncRNA metabolism, RNA metabolism, RNA secondary structure unwinding, RNA polyadenylation, translation, and ribosome biogenesis all had more genes induced than repressed in each category in roots (Figure 4.12B, 4.4C).
If RNA metabolism was the most dominant process among the boron stress-induced genes in *A. thaliana*, we hypothesized that the stress effect should be discernible at the entire transcriptome level. Therefore, we tested if the mean expression level per transcript for the entire transcriptome was significantly shifted in the excess boron-treated samples compared to the control, as previously described by Muyle and Gaut (2018). Excess boron stress did lead to an increased mean expression in *A. thaliana* roots and shoots and also in *S. parvula* shoots (Figure 4.12D). The boron stress-adapted *S. parvula*, however, did not show a mean expression change in roots implying its greater capacity to cope with excess boron without a massive change to its entire transcriptome. This may also suggest that the Arabidopsis global transcriptomic response has a significant energetic cost, which could also contribute to the delayed growth during excess boric acid treatments observed (Figure 4.1).

*A. thaliana* roots respond to excess boron by increasing the abundance of multiple amino acids, sugars, and nucleic acid-metabolites

We observed induction of genes especially associated with translation (Figure 4.12C) and ribosome biogenesis (Figure 4.12B) in *A. thaliana* roots, which was further supported by the increased average expression level observed for the entire *A. thaliana* root transcriptomes (Figure 4.12D). This led us to test whether changes in RNA metabolism and associated processes observed in the root transcriptomes of *A. thaliana* during excess boron stress affected amino acid usage. Additionally, we suspected that an increased level of translation may also lead to altered metabolic pools of sugars involved in primary energy metabolism especially in *A. thaliana* roots in response to excess boron.

The abundance of 32 functionally annotated metabolites changed significantly upon 5 or 10 mM boron treatments in *A. thaliana* roots. In contrast, none of these metabolites were affected by either boric acid treatment in *S. parvula* roots. Sugars and amino acids, and their derivatives,
constituted the two largest groups of boron stress-responsive metabolites in *A. thaliana* roots

Figure 4.12. RNA metabolism-related processes are affected in response to excess boron in *A. thaliana*. (A) Enriched functional clusters among boron stress-induced genes in *A. thaliana* roots with notable associations for RNA metabolism marked by red-dashed boxes. The network visualization is similar to Figure 3A. (B-C) Number of boron stress responsive genes from *A. thaliana* in functional categories associated with RNA metabolism (B) and translation (GO:0006412). (C). Red and blue indicate up- and down-regulation, respectively. (D) Global transcriptome expression distributions in *A. thaliana* and *S. parvula*. Density distributions of expression in log(RPKM+1) for entire transcriptomes were compared to identify transcriptome-wide global changes. p-values were estimated using Wilcoxon signed-rank test. (E) Major organic metabolic groups that changed their abundance in response to excess boron stress in *Arabidopsis* roots. For each category, the number and proportion of metabolites that changed in abundance compared to the total identified are shown. (F) Amino acid and (G) nucleic acid metabolite abundance changes in response to excess boron stress in shoot and root. The relative abundance is given compared to the internal standard, ribitol. Values shown are mean ± SD (n = 3, 4 or 5). Asterisks represent significant differences of each treatment compared to control according to Student’s t test (p < 0.05).
The relative abundance of 8 of the 13 amino acids detected significantly increased in the root tissues in response to the treatments (Figure 4.12F). This response was more prominent in roots than shoots, where only two out of the 10 amino acids detected changed significantly. The majority of sugars and their derivatives that responded to the treatments in the roots were primarily involved in glycolysis or sugar transport. For example, these included glucopyranose, fructofuranose, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, sucrose, and raffinose. This may be indicative of the generally higher demand for cellular energy consumption during induced transcription levels especially in A. thaliana roots under excess boron stress. Notably, the sugar-metabolite profile in the roots was quite distinct from the increased abundance of sugars in the shoots that were enriched primarily in cell wall precursors as described earlier (Figure 4.6D). Ribose, uracil, and adenosine that are related to nucleic acid metabolism also increased in abundance in shoots in response to excess boron, whereas only adenosine from that group increased in the roots (Figure 4.12G). Adenosine was the metabolite with the highest fold change in shoots and roots among all metabolites detected in our study (Figure 4.12G).

**Constitutive expression of S. parvula orthologs match the post-stress expression of A. thaliana boron stress-responsive orthologs**

To complement our studies focused on boron stress-sensitive A. thaliana, we next sought evidence for the types of biological processes that allow S. parvula to tolerate toxic amounts of boron. The overall transcriptomic, ionomic, and metabolomic responses elicited in S. parvula in response to excess boron were much less pronounced than those for A. thaliana. Nevertheless, we were able to observe enriched functions among the differentially expressed genes (Figure 4.13). Cell wall-modifying enzymes were the only enriched function observed for S. parvula
roots (Figure 4.13). Genes encoding protein modifying and mitochondria-localized proteins were also induced in response to the boric acid treatments in *S. parvula* shoots, while genes involved in biotic stress and defense responses and boron uptake were repressed in both roots and shoots (Figure 4.13).

Our comparative -omics framework allows us to gain insight into the *S. parvula* genes and processes that remained unchanged when their orthologs were differentially regulated in *A. thaliana* in response to excess boron. To this end, we compared the expression levels of orthologs from the two species under control and treated conditions. We identified ortholog expression for 19,263 pairs in shoots and 19,784 pairs in roots that were used to determine the co-expressed ortholog clusters. This led to 22 shoot and 20 root clusters. We further categorized those clusters into four overall expression trends that we have termed as, (a) stress-ready clusters; (b) unique-response clusters; (c) shared-response clusters; and (d) no-response clusters
Figure 4.14. Genes associated with excess boron responses are constitutively expressed in *S. parvula*. (A) Summary of co-expression trends in ortholog pairs between *A. thaliana* and *S. parvula* in response to excess boron. The red lines indicate the trend in expression levels of the ortholog pairs in each species under control and treatment conditions, compared to *A. thaliana* control (dashed line). Ctrl, Control; + B, boric acid treatment; S, Shoot; R, Root. (B) A major co-expression cluster in the “stress-ready” category in (A) that illustrates stress preparedness of the *S. parvula* orthologs in roots. (C) Functional clusters enriched among the ortholog pairs from the example cluster given in (B). The clusters are differently colored and labelled with the representative functions. Node size represents genes in the test set which are annotated to that functional term; edges represent the number of shared genes between functional terms; each cluster is coded with a different color; and shade of each node represents *p*-value assigned by the enrichment test. Lighter to darker shades indicate larger to smaller *p*-values, respectively. (D) *S. parvula* shows a higher basal level expression than *A. thaliana*. *p*-values were estimated using Wilcoxon signed-rank test.
stress to reach a level of expression equivalent to the basal level of the ortholog in the other plants, which itself remain unchanged under the stress. The unique-response clusters (b) represented ortholog pairs where one species showed a response that was unmatched in the other species either at the control or treated levels. Orthologs pairs with a similar response in both species to excess boron stress were categorized into the shared-response cluster (c). Finally, ortholog pairs that did not change their expression to excess boron stress were grouped as “no response” (d).

The majority of the ortholog pairs in the two species remained unchanged in response to excess boron (7,797 ortholog pairs from roots and shoots in the no-response group) (Figure 4.14A). When one ortholog in a pair did respond, the majority (~62%) of those showed only the expression change in the A. thaliana ortholog. The ortholog distribution in these categories further highlighted the more restrained transcriptomic responses of S. parvula and revealed an interesting but hidden feature of the S. parvula genome that we may not have identified without A. thaliana as a comparator. We did not identify a single ortholog pair where the S. parvula ortholog responded to the stress to reach the basal level of its A. thaliana ortholog (i.e. zero representation in the stress-ready group for A. thaliana). By contrast, we identified 2,160 A. thaliana orthologs whose expression changed to match the basal expression observed for the S. parvula orthologs. Additionally, we only identified 6 S. parvula orthologs that could be classified in the unique-response group (Figure 4.14A). This led us to propose that stress-adapted S. parvula had a pre-adapted transcriptome with over a thousand orthologs whose basal expression levels (pre-stress expression) match the expression levels achieved in response to the stress (post-stress expression) in stress-sensitive A. thaliana. Any differential expression shown by the orthologs in the stress-adapted species prompted by the stress was always echoed by the
stress-sensitive species. Thus, these cellular responses may be common among plants responding to excess boron and not restricted by species boundaries.

We also identified at least one thousand orthologs in *A. thaliana* roots and shoots that uniquely responded to excess boron. The expression of their *S. parvula* counterparts did not change significantly. We suspect that the majority of the expression changes in *A. thaliana* represent non-specific symptoms caused by interruption to cellular processes in a plant unable to sustain a cellular environment conducive for growth and development rather than a specific response to excess boron.

We also searched for enriched functions associated with the stress-ready clusters in *S. parvula* to determine what cellular or metabolic processes were enriched at stress-anticipatory levels in the basal transcriptomes. We first looked into the orthologs expressed in the *S. parvula* stress-ready category where *A. thaliana* orthologs were induced in response to excess boron (Figure 4.14B). These *S. parvula* orthologs were predominantly enriched for RNA metabolic processes (Figure 4.14C). It should be noted that the same enriched function was also the predominant function among all induced genes in *A. thaliana* roots regardless of their orthologous relationship with *S. parvula* (Figure 4.12A). We then compared the basal expression levels of all orthologs between *A. thaliana* and *S. parvula* to assess if the basal expression was significantly different between the two species. In both shoots and roots, the *S. parvula* transcriptome showed significant shifts towards overall higher gene expression levels compared to *A. thaliana* (Figure 4.14D). Taken together, these results suggested that *S. parvula* transcriptomes were pre-adapted for boron stress most notably in the metabolic functions associated with RNA metabolism that was among the most altered processes in the stress-sensitive *A. thaliana* during the excess boron treatments. However, in many of these stress-ready clusters, enriched functions only described a subset of
the orthologs, while a significant proportion of orthologs remained functionally uncharacterized (Figure 4.15).

![Figure 4.15. Number of members and their annotation availability in selected stress-ready clusters. RC: root cluster; SC: shoot cluster.](image)

**Discussion**

Combining our results and previous studies, we propose a model for how excess boron triggers transcriptomic responses that cascade into major cellular and growth responses (Figure 4.16). The stress-sensitive species, *A. thaliana*, in response to boron toxicity: 1) halts active boron uptake; 2) deposits a proportion of excess boron into cell walls; 3) adjusts the expression of genes involved in RNA metabolism; and 4) forms complexes with free boric acid, especially in roots. We demonstrated that boron toxicity induced minimal changes to gene expression, elemental and metabolite profiles, and growth in stress-adapted *S. parvula* when compared to *A. thaliana*. Different excess boron tolerance mechanisms are likely present in *S. parvula*. These include, 1) an efficient boron efflux system that minimizes excess boron accumulation in the plant; 2) cell wall absorption of a proportion of excess boron; 3) formation of B-complexes to reduce free boric acid accumulated in the cytoplasm before boron could bind to essential
metabolites; and 4) genes associated with cellular processes affected by excess boron in *A. thaliana* are constitutively expressed at stress pre-adapted levels.

*S. parvula* is equipped with an efficient boron efflux system

*S. parvula* is an extremophyte that has evolved to grow on boron-rich soils (Nilhan et al., 2008; Oh et al., 2014b). As expected, it was less affected by excess boron than the boron stress-sensitive model, *A. thaliana* (Figure 4.1). This is due in part to the ability of *S. parvula* to...
maintain relatively low boron levels in its tissues (Figure 4.1H). This is likely a feature of boron toxicity tolerance since other boron stress-tolerant plants, including *Eutrema salsugineum* (Lamdan et al., 2012) and *Puccinellia distans* (Stiles et al., 2010), also maintain a relatively low level of endogenous boron even when grown under excess boron conditions.

At physiological pH, boron primarily exists as uncharged boric acid, which is highly membrane permeable (Reid, 2014). Boric acid readily diffuses into the root cells under adequate or excess boron conditions (Yoshinari and Takano, 2017; Landi et al., 2019; Princi et al., 2016). Several mechanisms have evolved in plants to control boron influx and efflux. For example, *A. thaliana BOR4* encodes the only boron exporter experimentally shown to function under boron toxicity (Miwa et al., 2014, 2007). Surprisingly, we saw no significant change of expression of this gene in either species in response to excess boron. However, *BOR5*, the closest homolog of *BOR4*, was induced by excess boron in *A. thaliana* roots, and was highly expressed especially in the roots of *S. parvula* control plants (Figure 4.3C, D). This may be a result of a 15 kb transposition insertion in the upstream region adjacent to the *SpBOR5* transcription start site (Oh et al., 2014b).

*SpBOR5* and *AtBOR5* exist as single copy genes and are co-linear except for the genomic insertion in *S. parvula* (Oh et al., 2014b; Oh and Dassanayake, 2019). We demonstrated that *SpBOR5* is an effective boron exporter (Figure 4.3D) and propose that it is likely a key contributor to the underlying tolerance of *S. parvula* to excess boron (Figure 4.16).

**Excess boron taken into plants is differently compartmentalized in *A. thaliana* and *S. parvula***

The absorbed excess boron may exist in free or bound forms in plants. We observed that free boric acid levels increased in *A. thaliana* shoots and roots, as well as in *S. parvula* shoots as the external boric acid concentration increased (Figure 4.1H). Plants may attempt to minimize the deleterious effects of excess boric acid by exporting it to vacuoles. However, we saw no change
in the expression of TIP5;1, which encodes the only known aquaporin that facilitates boron transport into vacuoles (Pang et al., 2010), in either A. thaliana or S. parvula (Figure 4.17A). Other boron stress-responsive TIP genes all showed repression instead of induction in treated A. thaliana.

![Expression of TIP5;1 and CSLD5](image)

Figure 4.17. Expression levels of TIP5;1 (A) and CSLD5 (B) from A. thaliana and S. parvula in control and 5 mM boric acid treatment. Asterisks represent significant differences in expression compared to control (at FDR-adjusted p<0.05) determined by both DESeq2 and NOISeq.

In a previous study of two barley cultivars that differed in their boron tolerance, the boron stress-tolerant cultivar was reported to have a higher apoplastic boron content than in the sensitive cultivar (Reid and Fitzpatrick, 2009a). We found that the expression levels of AtBOR5 and AtBOR7 increased in A. thaliana roots in response to excess boric acid (Figure 4.3C). Therefore, it is possible that a proportion of excess free boric acid is exported into the apoplast, especially in A. thaliana roots. Consistent with this notion, previous studies have suggested that apoplastic boric acid constitutes the majority of soluble boron in plants under normal conditions, and even
in some species after exposure to excess boron (Matoh, 1997). The increase in free boric acid in
*A. thaliana* and *S. parvula* is unlikely to be the sole cause of the increased amounts of total boron
detected (Figure 4.1H and I). Rather, some absorbed boron must exist in a bound form especially
in *A. thaliana* roots and *S. parvula* shoots. The formation of B-complexes may have contributed
to the detoxification of excess boron. Alternatively, such complexes may also accumulate in the
cytoplasm as undesirable metabolic end products.

Our metabolomic profiles indicated that ribose increased in *A. thaliana* shoots under excess
boron (Figure 4.12G). This monosaccharide together with ribose-containing compounds,
including nucleotides, NADH, NAD+, and S-adenosylmethionine have the ability to form borate
esters in the cytoplasm (Ricardo, 2004; Ralston and Hunt, 2001; Kim et al., 2003, 2004). It is
notable that adenosine is among the largest metabolite changes (~65 fold increase) in treated *A.
thaliana* (Figure 4.12G). Boron could also form borate esters with sugar alcohols and organic
acids containing *cis*-diols (Bolanos et al., 2004). Several sugar alcohols, including galactinol,
erithritol, and cellobiotol increased substantially in treated *A. thaliana* shoots (Figure 4.6E). We
also observed that many unidentified compounds changed in *A. thaliana* during boron
treatments. Remarkably, none of the identified metabolites changed significantly in *S. parvula* in
response to excess boron treatments. This is consistent with our hypothesis of a transcriptome
pre-adapted to boron stress in the tolerant *S. parvula*.

The lack of substantial changes in the metabolite profiles of *S. parvula* led us to hypothesize two
possibilities for how it may minimize the cellular toxicity of excess boron in the cytoplasm. First,
generation of borate-containing metabolites may ameliorate toxicity but comes with a high
energy cost that would direct *S. parvula* to use more energy efficient alternative paths to store
excess boron. Second, if the generation of such B-complexes was harmful but unavoidable when
excess boron accumulated in the cytoplasm, *S. parvula* may prevent their accumulation by limiting the amounts of boron in the cytoplasm more efficiently than *A. thaliana*. When bound to boron, metabolites in the cytoplasm will be unavailable to critical primary metabolic processes. Thus, cells may attempt to increase the production of these metabolites at a rate that cannot be sustained in boron stress-sensitive species. The response of *A. thaliana* to increase many of these metabolites on excess boron are consistent with this view. Alternatively, mechanisms may have developed in *S. parvula* to process excess cytoplasmic boron in a manner that does not preclude ribose or other metabolite pools from functioning in their respective essential roles (Figure 4.16).

**Cell wall contributes to partially compartmentalize excess boron**

Several independent studies have provided compelling evidence for the existence of boron-rhamnogalacturonan-II (B-RG-II) complexes in plant cell walls (Kobayashi et al., 1996; Ishii and Matsunaga, 1996; O’Neill et al., 1996). There is also evidence that this complex is required for normal plant growth and development (Fleischer et al., 1999; Ishii et al., 2001; O’Neill, 2001). The carbohydrate–rich plant cell wall is ideally suited to bind boron (Matoh, 1997), but whether cell walls can store excess boron when plants encounter boron toxicity has not been demonstrated. Herein, we provide compelling evidence for this phenomenon. First, we found that while cell wall yield was unaffected, there was an increase in cell wall boron in *A. thaliana* shoots and roots, as well as in *S. parvula* roots, when plants were grown on excess boron (Figure 4.6F, G). Second, we have demonstrated that boron toxicity altered the expression of many genes involved in cell wall biogenesis or organization as well as pectin biosynthesis (Figure 4.6A, B). Third, our metabolomic profiling supported the transcriptomic signals related to the changes in the content of cell wall polysaccharide precursors, notably the monosaccharides used to synthesize pectin (Figure 4.6D). Together these observations strongly support the idea that cell
walls contribute, at least partially, to the compartmentation of excess boron in plants (Figure 4.16).

In line with our results, previous studies on *A. thaliana* and boron stress sensitive citrus cultivars showed boron accumulation in the cell sap-free tissue fraction when treated with excess boron (Lamdan et al., 2012; Martínez-Cuenca et al., 2015). A recent study of the trifoliate orange (*Poncirus trifoliata*) reported alterations in cell wall structure when plants were treated with excess boron (Riaz et al., 2019; Wu et al., 2019). In contrast to these findings, Dannel et al. (1998) suggested that cell walls did not absorb excess boron during boron toxicity based on studies of boron stress-resistant sunflowers. However, they did not quantify boron accumulation in tissues, and assumed that internal boron levels changed proportionally to the external boron supply; thereby ignoring the possible contribution of active extrusion of excess boron in plants. A subsequent study reexamined boron tolerance in sunflower and concluded that sunflower did exclude excess boron when compared to a sensitive species (Keleş et al., 2011). Several other studies, for example, have noted that barley roots (Hayes and Reid, 2004) and *Eutrema salsugineum* shoots (Lamdan et al., 2012) did not store excess boron in the corresponding cell walls. However, these studies did not include both roots and shoots when assessing how excess boron could be partly stored in certain tissues while some of it could be extruded back to the soil. In cell walls, boron can complex with apiose present in RG-II as well as with other sugars containing *cis*-diols (Matoh, 1997). Boron cross-linking of two RG-II molecules occurs rapidly during RG-II synthesis and secretion. Previous studies suggest that the crosslink is formed in the cytoplasm prior to RG-II deposition in cell wall rather than in the cell wall itself (Chormova et al., 2014; Chormova and Fry, 2016). *In vitro* assays have demonstrated that excess boron can reduce the rate of RG-II dimerization (Chormova et al., 2014). Therefore, future studies testing
the compositional changes of RG-II and other cell wall sugars during excess boron stress in plants could further identify how plant cell walls may be restructured to allow storage of excess boron.

**Boron toxicity disturbs RNA metabolism and related processes**

Excess boron resulted in substantial changes in the expression of genes involved in RNA metabolism and related processes, including translation and ribosome biogenesis (Figure 4.12A). Boron is known to form complexes with ribose (Ricardo, 2004) and ribose-containing compounds *in vitro* (Ralston and Hunt, 2001; Kim et al., 2003, 2004). Thus, one explanation for the extensive changes in RNA metabolism-related processes could be that excess boron affects the availability of ribose and ribose-containing compounds needed for RNA metabolism, and that creates a prominent transcriptional footprint.

Uluisik *et al.*, (2011) previously demonstrated that excess boron suppresses protein synthesis and interrupts translation initiation by reducing the proportion of functionally available polysomes in yeast. The authors further showed that excess boron also inhibits aminoacylation of tRNAs *in vitro*. Considering our transcriptomic and metabolomic results, together with the previous publications, it is reasonable to suspect that similar to yeast, excess boron in plants may impact protein synthesis by impairing polysome function. In addition, excess boron may also bind to the ribose moiety at the amino acid attachment site in tRNAs, which could block access to amino acids, thus inhibiting tRNA aminoacylation. In support of this view, our transcriptomic data shows that ribosome biogenesis was enhanced in *A. thaliana* roots and shoots after excess boron treatments (Figure 4.12B and 7).
**S. parvula transcriptome is pre-adapted to boron toxicity**

Compared to A. thaliana, S. parvula is more tolerant to boron toxicity (Figure 4.1). Our transcriptomic analyses suggest that S. parvula is pre-adapted for this stress (Figure 4.14A). While some of the S. parvula orthologs in the “stress-ready” cluster could be readily associated with enriched GO functions (Figure 4.13), not all orthologs could be represented by GO annotations inferred using experimentally established functions (Figure 4.15). The proteins encoded by many of these genes (>50% in stress-ready clusters) have no known functions described for their A. thaliana orthologs. This indicates a severe gap in the functional associations recognized between gene functions relevant to excess boron stress. Our comparative transcriptome analyses indicate that these genes of unknown functions in A. thaliana not only respond significantly to excess boron, but also their orthologs in S. parvula are expressed at levels comparable to the induced or repressed level in A. thaliana even in the absence of boron stress. Such stress-preparedness at the transcriptome level is likely a key contributor to the stress response in boron stress-tolerant plants. Indeed, similar transcriptome-level preadaptation to other abiotic stresses have been documented for plants that have evolved in environments where abiotic stresses are a constant feature (Taji et al., 2004; Gong et al., 2005; Becher et al., 2004b; Hassan et al., 2016).

**Why is excess boron toxic to plants?**

Our results demonstrated that when plants are grown in the presence of excess boron, some of this boron accumulates in cell walls. However, incorporating boron beyond an undefined threshold may trigger cell wall integrity signaling. We found >55% of genes (at least 300 in shoots and 150 in roots out of 628) coded for receptor-like kinases (RLKs) that responded to excess boron in A. thaliana. Many of these genes including wall-associated kinases (WAKs),
Catharanthus roseus RLK1 (CrRLK1)-like (CrRLK1L) kinases, and leucine-rich repeat (LRR) RLKs have been suggested to participate in cell wall integrity sensing (Steinwand and Kieber, 2010; Rui and Dinneny, 2019; Vahtera et al., 2019).

We observed that excess boron in A. thaliana shoots led to the repression of several cellulose synthases, including CesA2 and CesA3, and CesA like family members (CSLD5) (Figure 4.17B). CSLD5 is most highly expressed in the shoot meristem of A. thaliana and is required for initializing cell plate formation (Gu et al., 2016). Boron-dependent repression of CSLD5 may result in arresting cells in their G2/M transition phase, leading to cell division failures and growth defects. Further, excess boron is reported to decrease the number of mitotic cells and increase the fraction of 4C cells in A. thaliana root tips (Sakamoto et al., 2011). Additional studies have reported that inhibition of cellulose biosynthesis leads to the repression of cell cycle genes (Gigli-Bisceglia et al., 2018) and that key core cell cycle regulators are modulated by excess boron (Aquea et al. 2012). Our data are consistent with these publications, as we identified cell cycle processes together with exocytosis, which is related to cell-plate formation, as major functional groups among boron stress-repressed genes in A. thaliana shoots (Figure 4.18). Therefore, excess boron accumulation in cell walls may not only affect cell wall integrity, but also cell plate construction, which in turn may interrupt cell division. This may explain why the effects of excess boron become apparent in fast-dividing meristems before mature tissue (Choi et al., 2007; Reid et al., 2004; Aquea et al., 2012).

Excess boron is not only toxic to plants, but also to yeast and animals (Bakar Salleh et al., 2010; Bakirdere et al., 2014). Therefore, cell wall-mediated boron toxicity alone may not explain the toxic effects of excess boron on these systems, especially animal cells. Excess boron-associated DNA damage has been reported as a consequence of boron toxicity among eukaryotes.
(.Sakamoto et al., 2018). In addition, we showed that transcriptional signals related to RNA metabolism were substantially affected in A. thaliana, while S. parvula orthologs showed a stress-prepared expression level prior to the stress (Figure 4.12A, 6A, and 6B). We also observed transcriptome responses pointing to translation as a major target of boron toxicity. Similar results have been reported for yeast (Uluisik et al., 2011). Further, in human cells, excess boron increased the phosphorylation of eIF2α, which was inferred to lead to reduced protein synthesis (Yamada and Eckhert, 2018; Henderson et al., 2015).

In conclusion, we have shown that boron toxicity induces significant physiological and molecular changes in boron stress-sensitive A. thaliana compared to stress-adapted S. parvula. Excess boron accumulates in the cell walls of both shoots and roots, which may alter the structure and properties of the cell wall and its components. Such changes in the cell wall may affect cell plate formation, which in turn may lead to interruptions in cell division. Our data also suggest that boron toxicity interferes with RNA metabolism-related processes, especially translation, and other metabolic processes that involve ribose-containing metabolites. A model for how excess boron may trigger transcriptomic responses that cascade into major cellular and growth responses is presented in Figure 4.16. Further studies into cell wall dynamics during excess boron treatments in A. thaliana, as well as targeted functional analyses of A. thaliana stress-responsive genes that also show “stress-adapted” transcription in S. parvula to determine their currently unexplored functions would lead to an extended overview of how plants can survive excess boron stress.
Figure 4.18. Functional clusters enriched among genes that were induced (A) and repressed (B) in A. thaliana shoots. Top 10 largest clusters are differently colored and labelled with the representative functional terms. Each node represents a GO term; node size represents genes in the test set assigned to that functional term; GO terms sharing more than 50% of genes are connected with edges; and shade of each node represents the $p$-value assigned by the enrichment test (FDR-adjusted $p<0.05$) with darker shades indicating smaller $p$-values.
CHAPTER 5: PLANT GROWTH AND AGROBACTERIUM-MEDIATED FLORAL-DIP TRANSFORMATION OF THE EXTREMOPHYTE, SCHRENKIELLA PARVULA

Introduction

In this protocol we describe growth and establishment of stable transgenic lines for the extremophyte model, Schrenkiella parvula. The availability of an efficient transformation system is a hallmark of any versatile genetic model. Plants that thrive in extreme environments referred to as extremophytes, provide a critical resource for understanding plant adaptations to environmental stresses. Schrenkiella parvula (formerly Thellungiella parvula and Eutrema parvulum) is one such extremophyte model with expanding genomic resources (Dassanayake et al., 2011; Oh et al., 2012; Whited, 2015; Dassanayake et al., 2012; Dittami and Tonon, 2012). However, transformation protocols have not yet been reported for S. parvula in published studies.

The genome of S. parvula is the first published extremophyte genome in Brassicaceae (mustard-cabbage family) and shows an extensive overall genome synteny with the non-extremophyte model, Arabidopsis thaliana (Dassanayake et al., 2011). Thus, comparative studies between A. thaliana and S. parvula could benefit from the wealth of genetic studies performed on A. thaliana to make informative hypotheses on how the S. parvula genome has evolved and regulated differently to cope with extreme environmental stresses (Amtmann, 2009; Oh et al., 2014a; Dittami and Tonon, 2012). S. parvula is one of the most salt-tolerant species (based on soil NaCl LD50) among known wild relatives of A. thaliana (Orsini et al., 2010). In addition to the NaCl tolerance, S. parvula survives and completes its life cycle in the presence of multiple

salt ions at high concentrations toxic to most plants (Oh et al., 2014b). In response to the abiotic stresses prevalent in its natural habitat, it has evolved various traits among which several have been studied at the biochemical or physiological level (Uzilday et al., 2015; Teusink et al., 2002; Jarvis et al., 2014; Orsini et al., 2010).

Since 2010, there have been over 400 peer-reviewed publications that used S. parvula as a target species or used it in a comparison with other plant genomes. However, a clear bottleneck could be identified with a closer look of what type of studies have been conducted. The majority of these reports discuss the potential use of S. parvula in future studies or use it in comparative genomic or phylogenomic studies. Due to the lack of a proof-of-concept transformation protocol established for S. parvula, it has not been used in functional genomic studies, despite having one of the highest quality plant genomes available to date (>5Mb contig N50) assembled and annotated into chromosome-level pseudomolecules (Dassanayake et al., 2011).

The Agrobacterium-mediated floral-dip transformation method has become the most broadly used method to create transgenic lines in A. thaliana and the development of a reproducible system of transformation was a critical factor in its success as a genetic model (Clough and Bent, 1998; Koornneef and Meinke, 2010). However, not all Brassicaceae species have shown to be successfully transformed using the floral-dip method developed for A. thaliana. Specially, the Brassicaceae Lineage II species that include S. parvula has been recalcitrant to floral-dip based transformation methods (Bai et al., 2013; Sparrow et al., 2011).

The indeterminate flowering growth habit of S. parvula, combined with its narrow leaf morphology has made it challenging to adopt the standard Agrobacterium-mediated floral-dip transformation method. In this study, we are reporting the modified protocol we have developed for reproducible transformation of S. parvula.
Protocol

1. Plant growth

1.1 Seed sterilization (optional)

1.1.1 Prepare 50% bleach in double-distilled water (ddH₂O) with 1 or 2 drops of Tween 20 in a 50 mL tube. Invert the tube several times to mix the solution.

Note: It is preferable to conduct seed sterilization in a laminar flow cabinet with a UV sterilized surface for 15 minutes.

1.1.2 Add the bleach solution to ~100-200 S. parvula seeds in a 1.5 mL tube. Mix thoroughly and let the tube sit for 5 minutes.

1.1.3 Remove the bleach from the tube and add 70% ethanol. Wash the seeds by pipetting several times and then remove the ethanol solution immediately.

1.1.4 Wash the seeds in sterilized water to remove excess bleach and ethanol and then remove the water. Repeat this step for 5 to 6 times.

1.2 Seed stratification

1.2.1 Immerse the seeds in sterilized water, and store for 5 to 7 days at 4 °C. Alternatively, sow dried unsterilized seeds directly on wet soil, and place the soil tray for 5 to 7 days at 4 °C.

1.3 Growing plants in preparation of transformation

1.3.1 Fill soil into 7 x 6 cm² pots; soak the pots in water; and spray water from the top to ensure a uniformly wet growth medium. Add 5-6 Osmocote (Scotts Miracle-Gro Co., Marysville, OH) fertilizer beads on the soil surface of each pot.

1.3.2 Using a wet toothpick, transfer 20~25 seeds per pot on the soil surface. Note: A convenient practice is to put a batch of 4-5 seeds in the four corners and the center of the pot.
1.3.3 Cover the pot tray with a clear dome to keep the seeds under high humidity during germination.

1.3.4 Keep the plant trays in a growth chamber with a light intensity set at 130 µmol m\(^{-2}\) s\(^{-1}\) light, 22 – 24 °C temperature, and 14-h-day/10-h-night cycle. Remove the domes after 7 – 10 days following germination. Add water from the bottom of the tray to keep soil moistened uniformly at a desirable level.

1.3.5 Weed out extra seedlings and leave only 4-5 healthy seedlings per pot well separated from each other.

1.3.6 Gently water the plants every two days and fertilize with 0.2x Hoagland’s solution (Hoagland and Arnon, 1950) once every two weeks. Note: Keeping the soil moisture at a uniform level is key to growing *S. parvula* consistently and healthily.

1.3.7 Continue to grow the plants for 8-10 weeks until multiple inflorescences produce 100-150 floral buds per plant. On the day planned for the floral-dip based transformation (step 4.5), remove all mature and developing siliques from the plants.

2. Cloning the gene/genomic element of interest into competent *Escherichia coli*

2.1 Amplify the target DNA fragment using polymerase chain reaction (PCR (Saiki et al., 1988)) and sequence (Sanger et al., 1977) the purified PCR product for verification of the target sequence. Gel extraction of PCR product is done with MEGAquick-spin Total fragment DNA purification kit, iNtRON biotechnology.

2.2 Setup the TOPO cloning reaction to insert the desired PCR product into the pENTR TOPO vector (Gateway cloning technology, Invitrogen Co.) following manufacturer’s guidelines.

2.3 After performing the TOPO cloning reaction, transform the pENTR TOPO construct into NEB 5-alpha *E. coli* competent cells following manufacturer’s guidelines.
2.4 Pour 50 µL of transformed products in LB (Bertani, 1951) plates with appropriate antibiotics (Spectinomycin 50 µg/mL) and keep at 37 °C for overnight to develop colonies. Note: After autoclaving the LB media, allow it to cool to 55-60 °C before adding antibiotics to the media and pour into plates.

2.5 The following day, select 5-10 single colonies and transfer into liquid LB medium with appropriate antibiotics (Spectinomycin 50 µg/mL). Place the tubes on a shaker for overnight at 37 °C.

2.6 Extract the plasmid using Zippy Plasmid Miniprep Kit (Zymo Research) and sequence to verify that the target sequence was amplified and correctly inserted as described in step 2.1. For the sequence confirmed bacterial colonies, make a glycerol stock for future use in a 2 mL screw-cap tube and store at -80 °C. A glycerol stock is made by mixing 500 µL of bacterial culture and 500 µL of 50% glycerol. 50% glycerol is made by mixing equal volumes of 100% glycerol and ddH2O.

2.7 The sequence confirmed cloned fragment is used to perform a recombination reaction using the Gateway LR ClonaseII enzyme mix. This is to perform the multi-site gateway LR recombination reaction to introduce the gene of interest into the appropriate destination vector. Example destination vectors include pBGWFS7 and pB7WG2 for cloning promoters and overexpression of target genes respectively. Both of these destination vectors are Gateway compatible and Spectinomycin can be used to screen for transformed bacteria.

2.8 Transform the reaction mix from step 2.7 into NEB 5-alpha E. coli competent cells following manufacturer’s guidelines. Repeat the step 2.4 to 2.6 for introducing the gene/promoter of interest into the destination vector.

3. Transformation into Agrobacterium tumefaciens
3.1 Transform the target DNA construct into *A. tumefaciens* strain GV3101:pMP90RK, which Rifampicin can be used for chromosomal background selection and gentamycin or kanamycin can be used to select for the Ti plasmid. A brief protocol for electroporation is included in section 3.2.

3.2 *A. tumefaciens* electroporation

3.2.1 Take 1-2 µL of the PCR product of the sequence confirmed insert in the pDEST plasmid.

3.2.2 Thaw the *A. tumefaciens* competent cells on ice. Mix plasmid and competent cells on ice. Transfer the mixture into a 0.2 cm electroporation cuvette (USA Scientific).

3.2.3 Perform the electroporation reaction by selecting the *A. tumefaciens* mode on the electroporator (MicroPulser, BIO-RAD) following the instrument user manual guidelines. Note: clean the surface of the cuvette before starting the electroporation.

3.2.4 Transfer the reaction mixture from the cuvette to a microcentrifuge tube that contains 1.5 mL liquid LB, and mix well with pipetting.

3.2.5 Place the transformed product on the shaker for 1 hour at 28 °C.

3.3 Grow the transformed *A. tumefaciens* from section 3.2 on LB plates containing appropriate selection antibiotics (kanamycin 25 µg/mL, spectinomycin 50 µg/mL, gentamycin 25 µg/mL, and rifampicin 50 µg/mL) and keep at 28 °C for 3 days.

3.4 Amplification and selection of positive *A. tumefaciens* transformants

3.4.1 Select the single transformed colonies from plates and dissolve in LB liquid media containing appropriate antibiotics (kanamycin 25 µg/mL, spectinomycin 50 µg/mL, gentamycin 25 µg/mL, and rifampicin 50 µg/mL) to grow for another 3 days at 28 °C placed on a shaker. Note: Store the plate at 4 °C for future use.
3.4.2 Check the bacterial growth using a nanodrop (or a spectrophotometer) to obtain an optimal growth stage of \( \text{OD}_{600} \) around 2.0.

3.5 If the construct can be confirmed, make a glycerol stock of the \textit{A. tumefaciens} culture as described in section 2.6.

4. **Agrobacterium-mediated transformation of \textit{S. parvula}**

4.1 Take a single colony from the plates stored at 4 °C or ~20 µL from the \textit{A. tumefaciens} glycerol stock and dissolve in 10 mL of liquid LB medium in 50 mL falcon tubes containing appropriate selection antibiotics (kanamycin 25 µg/mL, spectinomycin 50 µg/mL, gentamycin 25 µg/mL, and rifampicin 50 µg/mL) and incubate at 28 °C on a shaker for 36 hours. Add additional LB solution to make the final volume 40 mL and incubate the culture for another 36 hours.

4.2 Centrifuge the \textit{A. tumefaciens} culture at 3100 × g for 10 minutes to form a pellet.

4.3 Remove the supernatant and add 40 mL of \textit{A. tumefaciens} infiltration solution to dissolve the bacterial pellet completely.

4.4 Dilute the bacterial solution with infiltration solution to a final \( \text{OD}_{600} \) value of ~0.8, measured with a nanodrop.

4.5 Add 25 µL of Silwet L-77 to 50 mL of bacterial solution from section 4.4 and mix the solution by inverting the tubes for several times.

4.6 Dip the inflorescence of the plants in the bacterial solution for 20 s with gentle agitations. Use a fresh bacterial solution after using one batch for six pots. Make sure all the flowers are dipped in the solution.

5. Plant growth after transformation
5.1 Place the floral-dipped plants horizontally in clean trays with domes to cover the plants and place in a dark growth room for 1-2 days. Note: Keeping the flowers under high-humidity is important at this stage.

5.2 Return the plants to an upright position and transfer the plants to a growth room with a 14-h-day/10-h-night cycle, 130 µmol m\(^{-2}\) s\(^{-1}\) light intensity and 22 to 24 °C temperature.

5.3 Monitor the dipped inflorescences in the following week. If a significant number of flowers abort, repeat the floral dip (step 4).

5.4 Grow the plants until seeds mature and harvest seeds at ~21 weeks.

5.5 Dry seeds for 2-3 weeks at room temperature in an airtight container (Dry Keeper, Sanplatec Corp) with anhydrous calcium sulfate (Drierite).

6. Selection of positive transformants

6.1 Plant the T1 seeds as described for wild type seeds in steps 1.2 to 1.3.

6.2 Grow the plants until the first 2-3 true leaves develop, in approximately 10 - 14 days after germination.

6.3 First selection for BASTA resistance by spraying

6.3.1 Spray pre-diluted (1:1000 v/v) BASTA on the seedlings and cover the plants with domes overnight.

6.3.2 Repeat BASTA spraying 2-3 times every 5-7 days.

6.4 Second selection for BASTA resistance using a drop test

6.4.1 Identify the plants that survive after 3-4 times of BASTA spray.

6.4.2 Grow the plants for another 2-3 weeks until 3-5 leaves develop a relatively larger leaf area.
6.4.3 Select the largest mature leaf per plant and place a drop of BASTA on that leaf selecting one leaf per surviving plant.

6.4.4 Monitor the leaves that had a BASTA drop for one week.

6.4.5 Keep the plants that had leaves unaffected by the BASTA drops.

6.5 Confirmation of positive transformants

6.5.1 Collect 2-3 leaves from the surviving plants at step 6.4.5.

6.5.2 Extract genomic DNA from the leaves using the CTAB method (Murray and Thompson, 1980) or any other appropriate DNA extraction method.

6.5.3 Perform PCR using extracted genomic DNA from target plants, wild type genomic DNA (used as a negative control), and plasmid construct with the \textit{bar} gene transformed to \textit{Agrobacterium} (used as a positive control), using \textit{bar} specific primers.

6.5.4 Confirm the presence of the expected size of the amplified \textit{bar} PCR product by agarose gel electrophoresis for the target samples.

6.5.5 Confirm the sequence of the amplified PCR product from target samples by sequencing.

\textbf{Representative results}

We developed a transformation protocol that enables harvesting of $T_0$ seeds within 150 days, using a floral-dip method modified from that for \textit{A. thaliana}. Figure 5.1 shows a summary of the timeline and \textit{S. parvula} plants that represent the optimal stage for executing the transformation through floral-dip. We selected \textit{S. parvula} plants with 70-80 flowers in multiple inflorescences at 60 – 80 days after germination as the target stage for transformation. A small number of pre-existing open or fertilized flowers and siliques at this stage were removed before the infiltration of \textit{A. tumefaciens} by the floral-dip method. Infection with \textit{A. tumefaciens} resulted in abortion of some flowers (Figure 5.2, bracket (a)). Siliques fully developed after the floral-dip are likely to
Figure 5.1. Timeline of *S. parvula* transformation. Keep seeds at 4 °C in water or on damp soils for 5~7 days to ensure even germination (Day 7). Plant 4~5 seeds on five spots in a pot (the four corners and the center), allow them to germinate and grow for a week, and weed out extra seedlings and leave one seedling per each spot (Day 15). Perform the transformation by flower-dipping when each plant develops multiple inflorescences and contain 100~150 flowers (Day 60~80). Part of flowers may have already developed to siliques, which need to be removed before the transformation. Keep plants moist under a cover after the transformation. Plants can be applied with a second round of transformation, with a two to three-week interval. Afterwards, allow the majority of siliques fully mature (Day 130) and harvest the T₀ seeds (Day 150).
contain transformed seeds (Fig. 5.2, bracket (b)). Even after transformation, *S. parvula*

![Image of S. parvula plants after transformation by flower dipping. (A) Plants photographed 10 days after the first flower dipping at Day 60. (B) Plants photographed 25 days after the second round transformation at Day 85. Note that infiltration with *Agrobacterium* may abort silique development of flowers as shown in brackets a. Siliques fully developed after flower dipping are likely to contain transformed seeds (brackets b). White arrows indicate flowers and inflorescences newly emerged after each transformation. *S. parvula* exhibits an indeterminate flowering habit, i.e., the plant keep producing new inflorescences and flowers until the end of the life cycle.

continued to develop new inflorescences and flowers as long as the plants are kept healthy (Fig. 5.2, white arrows). Due to this indeterminate flowering habit, a second round of transformation can be performed if the plant does not show signs of stress or senescence. Fig. 5.2A and 2B show examples of *S. parvula* plants after the first and second round of transformation, respectively, 25 days apart from each other. In the second transformation, existing siliques should not be removed because they may contain transgenic seeds. Also, the *A. tumefaciens* can be applied by pipetting the infiltration solution (Table 5.1) onto newly emerging flower clusters, instead of
dipping the entire shoot into the solution, to minimize the damage to siliques from the first transformation.

Table 5.1. The composition of solutions used in the transformation protocol.

<table>
<thead>
<tr>
<th>Luria-Bertani (LB)-bacterial growth media</th>
<th>Agrobacterium infiltration solution</th>
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<tbody>
<tr>
<td>Reagent</td>
<td>Amount</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g (plates only)</td>
</tr>
<tr>
<td>ddH2O</td>
<td>955 mL</td>
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<td></td>
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Figure 5.3. Selection of *S. parvula* transformants based on BASTA resistance. (A) T1 seeds are germinated approximately 70 seeds per pot. (B) BASTA sprayed a week after germination. (C) BASTA drop test is conducted on survivors after 3-4 weeks to identify true positives. False positives (top panel) are removed and true transgenic (lower panel) plants are left to reproduce.

The transformation efficiency is 0.033% yielding 3-4 transgenic plants per 10,000 T1 seeds.
propagated using the current protocol. This estimate is based on ~50,000 T1 seeds tested during ten independent transformation attempts. The multiple BASTA spray and drop tests will be critical to identify true positive transformants compared to false positives. Step 6.3 and 6.4 will also enable less false positive targets needed to be tested using the PCR confirmation in step 6.5

Figure 5.4. Confirmation of *S. parvula* transformation. (A) Genomic PCR amplification of *bar* gene in transgenic *S. parvula* plants. Lanes 1 and 13: size markers; Lane 2: negative control; Lanes 3, 4, 5: wild-type *S. parvula*; Lanes 6, 7, 8, 9, 10: transgenic *S. parvula* candidates; Lanes 11, 12: vector control. Lanes 7, 8, and 9 were determined as positive transformants. (B) An example of GUS expression in a positive *S. parvula* transformant.
Further confirmation of transformation can be checked with a reporter gene expression if the cloned sequence includes a reporter gene (Figure 5.4).

**Discussion**

The physiological state of the plant significantly influences the efficiency of transformation (Ghedira et al., 2013). The use of healthy and vigorous plants for transformation is a key requirement for successful transformation in *S. parvula*. Water or light stressed plants will have fewer flowers compared to the healthy plants ideal for transformation (Figure 5.1. center panel). *S. parvula* can grow at a light intensity less than 130 µmolm$^{-2}s^{-1}$, but the plants tend to be more frail and such plants would lead to more aborted flowers following floral-dip. *S. parvula* tends to abort Agrobacterium-dipped flowers at a higher rate than *A. thaliana*. Therefore, every step taken to minimize aborted flowers when dipped in the *A. tumefaciens* infiltration solution contributes to a higher transformation efficiency. We recommend a light period no longer than 14 hours per day. Often, transformation of *A. thaliana* is performed on plants grown in a long-day condition (e.g. 18-hour light and 6-hour dark) or even under continuous light. However, we found such practices result in less resilient *S. parvula* plants and lead to a low transformation efficiency. Flower buds are continuously produced on the inflorescence axes of *S. parvula* (Figure 5.2. White arrows). Therefore, allowing transformation of new flowers would significantly increase the chance of getting positive transformants. A second floral-dip (step 5.3) is not essential, but strongly suggested. However, this step is relatively time consuming compared to *A. thaliana* floral dipping, because *S. parvula* produces multiple inflorescence axes.

Wild-type *S. parvula* is sensitive to BASTA, although the initial screen for positive transformants with BASTA spray (step 6.3) will leave 5-8 surviving plants out of 100 seeds germinated (Figure 5.3A and B). Most of this (>80%) would be false positives. This is largely
due to the narrow leaf shape and the leaf angle of S. parvula that do not provide sufficient leaf surface in an appropriate orientation to retain the BASTA solution for a sufficient duration to observe a phenotype. Additionally, due to the high wax content of the adaxial leaf surface of S. parvula (Teusink et al., 2002), it tends to create a more impervious surface for BASTA. Therefore, the second screening for positive transformants using a BASTA drop on individual leaves (step 6.4) (Figure 5.3C) is an essential step to avoid PCR testing on hundreds of false positives (step 6.5).

The current protocol was tested with the A. tumefaciens strain GV3101 carrying the pMP90RK plasmid. The efficiency of transformation maybe improved with other A. tumefaciens strains including strains ABI, LMG20, and C58C1 Rifr with pMP90 virulence plasmid reported to increase transformation efficiency in A. thaliana (Ghedira et al., 2013). Brassica and Eutrema species are taxonomically more closely related to S. parvula compared to A. thaliana (Dassanayake et al., 2011). Therefore, the A. tumefaciens strain LBA4404, that was successfully used to transform Brassica napus and the strain EHA105 that has been used successfully to transform Eutrema salsugineum may offer a higher transformation efficiency than the reported efficiency of the strain currently used (Shaohong et al., 2005; Li et al., 2010, 2007).

Reducing the time and labor required by a transformation protocol is another significant factor in improving the transformation efficiency. Placing individual BASTA drops on leaves and monitoring the leaf for a week on multiple plants (step 6.4) are tedious. A future effort to increase the transformation efficiency could search for appropriate alternative selectable marker genes (Wu et al., 2015).

The availability of an established transformation protocol will greatly advance our ability to identify genes and novel mechanisms that allow extremophyte model plants to survive multiple
abiotic stresses (Oh et al., 2012; Dassanayake et al., 2012). Novel genetic variation in *S. parvula*
will provide a broader pool of genetic variation that cannot be mined from the collective allelic
variation identified as stress-responsive genes in the relatively stress-sensitive model, *A. thaliana*
pan-genome (Amtmann, 2009; Dittami and Tonon, 2012). Therefore, our floral-dip based *A.
tumefaciens* mediated transformation protocol developed for *S. parvula* would fill a gap for the
need for such tools to perform functional genomic experiments in an extremophyte model
closely related to *A. thaliana*. 
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

My work adds to a series of explorations that started more than a century ago in 1899 by the eminent botanist Edwin Copeland who first described the devastating effects of excess boron in plants. Boron toxicity poses a significant challenge to global agriculture. Understanding how plants have evolved to survive these stresses is imperative to plan for sustainable agriculture especially during a climate crisis. Our limited understanding on how plants respond, survive, and adapt to excess boron has been a major hurdle to engineer boron stress tolerance in plants using modern tools in plant breeding. As boron stress often co-exists with salinity and drought (Reid, 2010), the lack of knowledge on boron toxicity also complicates the selection and engineering of crops that can simultaneously survive these combined stresses. Thus, an improved understanding on boron toxicity in plants is critical to design future crops customized for different environments towards sustainable global food security.

In this dissertation, I sought to provide mechanistic insight into plant boron toxicity responses and tolerance by comparing a boron stress-sensitive model, *A. thaliana* and a boron stress-tolerant model, *S. parvula* in Brassicaceae in a multi-omics framework to deduce the overarching genetic and cellular processes underlying physiological responses. Boron stress is known to inhibit plant growth and promote cell death. As expected, *A. thaliana* showed reductions in plant biomass, chlorophyll content, and root growth, whereas *S. parvula* remained largely unaffected when I grew both species in comparable excess boron treatments. In the subsequent transcriptomic, metabolomic, and ionomic analyses, several key cellular responses, among which cell wall and RNA metabolism related processes emerged as primary targets of excess boron toxicity. I developed both computational tools and a species-optimized transformation method to aid the overall investigation of boron tolerance in plants.
Upon high external boron levels, the stress sensitive species allow dramatic increases in total boron as well as free boric acid content in plant tissues. Consequently, increased internal boron levels led to significant expression changes of one third of protein coding genes in the *A. thaliana* genome, indicative of massive changes at the transcriptomic level. Among these boron stress-responsive genes, processes related to cell wall metabolism was identified as one of the two predominant cellular functions affected by excess boron. GOMCL, a bioinformatics toolkit I developed in the process of analyzing the transcriptome data was influential in summarizing representative gene functions from large gene clusters (Wang et al., 2020b). Gene expression changes related to cell wall metabolism in response to excess boron stress in *A. thaliana* collectively pointed to a potential increase in pectin and especially RG-II biosynthesis.

Supportive of this observation, our metabolite profiling showed increases of many cell wall polysaccharide precursors, including those preferentially used for RG-II-pectins. In addition to the genes involved in cell wall metabolism, excess boron altered the expression of key genes for cell plate formation, cell cycle, and exocytosis. Previous studies have shown that plant meristems are often affected by excess boron before mature tissues where cell division is primarily taking place (Choi et al., 2007; Reid et al., 2004; Aquea et al., 2012). It should be noted that the primary role of boron in plant tissues is to provide cross-linking of RG-II dimers in plant cell walls (Ishii et al., 1999; O’Neill, 2001; Funakawa and Miwa, 2015). Taken these together, I propose that cell plate formation might be one of the major cellular sites where excess boron exerts its toxic effects in plants when cell wall pectin biosynthesis cannot be matched to the need to sequester more excess boron in cell walls. In support of this view, *A. thaliana* sequesters some of the excess boron in cell walls (more pronounced in shoots), but with prolonged stress show growth inhibition and eventually necrotic tissue.
An equally dominant process that was perturbed in *A. thaliana* deduced from transcriptomic signatures was RNA metabolism-related processes, especially translation. In congruence with the boron stress-responsive metabolic transcriptomic profiles, more than half of the detected amino acids in our metabolomics profiles showed changes in their abundances under boron stress. Previous studies have shown that free boric acid can bind to ribose or ribose containing metabolites (Ricardo, 2004; Ralston and Hunt, 2001; Kim et al., 2003, 2004). Therefore, I proposed that the primary cytotoxic effects of boron are elicited by excess boron binding to ribose-containing metabolites making them unavailable for critical cellular processes such as ribosome-driven translation.

Compared to *A. thaliana*, *S. parvula* exhibited much lower excess boron accumulation. Boron primarily diffuses into root cells under adequate and excess boron conditions at physiological pH (Yoshinari and Takano, 2017; Landi et al., 2019; Princi et al., 2016). I hypothesized that *S. parvula* is equipped with an efficient boron efflux system. Therefore, in my search for potential contributors for boron efflux, BOR5, a homolog of the BOR4 transporter that showed over 2000-folder higher basal expression in *S. parvula* roots compared to its *A. thaliana* ortholog was selected as a promising candidate. The heterologous expression of *BOR4* and *BOR5* from both species in yeast confirmed the greater capacity of *SpBOR5* to exclude boron as an efficient transporter. I have further characterized *SpBOR5* in *S. parvula* using promoter-reporter constructs have confirmed its preferential expression in roots where it would be able to exclude some of the excess boron back into soil (Figure 6.1). The *SpBOR5* transgenic lines were developed using the protocol developed for *S. parvula* as an effort to expand its molecular toolkit for functional genomic analyses (Wang et al., 2019). Further characterization of *SpBOR5* using overexpression and CRISPR editing is ongoing.
When comparing orthologs between *S. parvula* and *A. thaliana*, 38% of boron stress-responsive *A. thaliana* orthologs matched the pre-stress condition found in the corresponding *S. parvula* orthologs. This stress-preparedness at the transcriptomic level represented another strategy key strategy *S. parvula* exhibited to cope with boron stress. In line with previous findings primarily based on the *A. thaliana* transcriptomes from stressed samples, the stress-adapted transcripts in *S. parvula* were enriched in RNA metabolism-related process. This further strengthened the argument for ribosome-driven processes being key targets of boron toxicity. At a broader scale, *S. parvula* showed an increased average expression per transcript compared to *A. thaliana* and the average expression per transcript in both *A. thaliana roots* and shoots increased in response to excess boron exemplifying the preadapted signature for *S. parvula* transcriptomes.

The current work improves our understanding of boron toxicity in plants by highlighting key cellular processes impacted by excess boron and identifying genetic innovations in stress-adapted species which have evolved to cope with high soil boron levels. The tools, both bioinformatic and molecular, developed in this dissertation can be used in broader studies exploring other abiotic stress adaptations found in extremophytes, and especially the computational pipelines used in this project can be used or adapted into diverse comparative studies. I propose quantification of RG-II content to determine the ratio of RG-II dimers to monomers in cell walls from *A. thaliana* and *S. parvula* during excess boron treatments; assessment of excess boron on cell plate formation in dividing cells and meristematic tissue; polysomes quantification during excess boron stress; and heterologous expression of *S. parvula* boron transporters in *A. thaliana* and crops such as rice or tomato as next steps in the continuation of this investigation. Further studies into cell wall dynamics in boron stress-sensitive species, as well as targeted functional analyses of *A. thaliana* stress-responsive genes
that also show “stress-adapted” transcription in stress-adapted species to determine their currently unexplored functions would lead to an extended overview of how plants can survive excess boron stress.

Figure 6.1. Localization of proBOR5-GUS in ~10 day-old S. parvula carrying pSpBOR5::eGFP::GUS. (A) whole seedling; (B) cotyledons and leaves; (C) hypocotyl and root junction; (D) primary root; (E) lateral root. GUS staining was observed at true leaves and root in the transgenic S. parvula expressing proBOR5-GUS, especially at hypocotyl and root junction, root tips.
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VITA

Guannan Wang, son of Aiju Li (deceased) and Weixing Wang, is a native of China. He was born in 1990 in Mengzhou, Henan, where he spent much of his childhood with his family. He attended Henan Agricultural University for his Bachelor’s degree in 2009, and completed college in three years, one year ahead of the schedule. During undergraduate studies, he worked on wheat stripe rust under the supervision of Dr. Wenming Zhen. In August 2014, he joined Dr. Dassanayake’s lab at Louisiana State University to pursue his Ph.D. His graduate work included investigating the molecular mechanisms of plant responses and adaptations to boron toxicity. He anticipates graduating with his Ph.D. degree in Biological Sciences in December 2020. He plans to continue promoting LSU for many years to come.