Analysis of genes that respond to boron deficiency in model and crop plants

(ホウ素欠乏に応答する遺伝子のモデル植物や穀物を用いた解析)

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CONTENTS

List of Figures and Tables……………………………………………..……6

List of abbreviations in this thesis……………………………………....10

OUTLINE…………………………………………………………….…11

Chapter 1: Introduction……………………….………………….…..…12

1.1 Properties and distribution of B in nature…………………………..…..……12

1.2 The physiological role of B in plant……………...……………………………14

1.2.1 Role of B in cell wall structure……….………………………………………14

1.2.2 Role of B in membrane function……………………………………………...15

1.2.3 Role of B in metabolic pathways……………………………………………...16

1.3 B uptake and transport in plant under B limitation...…………………..………19

1.3.1 B uptake by root plants…………………..…………………….……………19

1.3.2 B transport in plants……………………………………………………………21

1.4 B deficiency response in plants……………………………………………...23

1.4.1 Physiological response of plant to low B…...…………………………………23

1.4.2 B deficiency in wheat……………………………………………………….…24

1.4.3 B deficiency in rice……………………………………………………….…25

1.4.4 B deficiency in maize…….……………………………………………………26

Chapter 2: Differential expression of three *BOR1* genes corresponding to different genomes in response to B conditions in hexaploid wheat (*Triticum aestivum* L.)……………………………………...…………….28

2.1 Introduction………………………………………………………………….28

2.2 Materials and Methods…………………………………………………...30

2.2.1 Plant materials and cultures………………………………………………..30

2.2.2 Sequence alignment and phylogenetic analysis………………………………30

2.2.3 Plasmid constructions (by Dr. Takahiro Fujibe)……………………………. 31

2.2.4 Determination of Ta BOR1s activity in BY-2 cells (by Dr. T. Fujibe)………32

2.2.5 Subcellular localization of Ta BOR1s-GFP (by Mr. S. Wang)………………32

2.2.6 RNA extraction and quantitative real-time PCR……..………………………33

2.3 Results……………………………………………………………………...…35

2.3.1 Phylogenetic analysis of Ta BOR1s...……………………………..………35

2.3.2 B transport activity of Ta BOR1s (by Dr T. Fujibe)…….…………………...…41

2.3.3 Subcellular localization of Ta BOR1.1 in *A. thaliana* leaf cell (by Mr. S. Wang)...43

2.3.4 Patterns of Ta *BOR1*s transcript accumulation: tissue specificity and response to B conditions..45

2.4 Discussion……………………………………………………………………..…48

2.4.1 Ta BOR1s are orthologs of Os BOR1 and At BOR1…..……………………48

2.4.2 The expression of three Ta *BOR1* in roots and shoots are differentially affected by B conditions……………..………………………………………………...49

2.4.3 Possible functional differences among the Ta BOR1s……………………..…50

Chapter 3: Comparison of *BOR1*-like gene expression in two genotypes with different B efficiencies in wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.) in Thailand……………..…………53

3.1 Introduction………………………………………………….………………53

3.2 Materials and methods……………………………………………………….53

3.2.1 Plant materials and growth condition……………..………………………54

3.2.2 RNA extraction …………………………………..……………….………55

3.2.3 Quantitative real-time PCR……………………….………………………55

3.3 Results…………………………………………..……..……………………57

3.3.1 Transcript levels of Ta *BOR1s* genes among two genotypes of wheat related to B deficiency…………………………………………………………………57

3.3.2 Transcript levels of Zm *BOR1* gene among two genotypes of maize related to B deficiency……………………………………………………………65

3.3.3 Transcript levels of Os *BOR1* gene among two genotypes of rice related to B Deficiency…………………………………………………………...69

3.4 Discussion………………………………………………………….………………72

3.4.1 The expression levels of Ta *BOR1s* genes correlate with the ability of B deficiency tolerance in wheat……………………………………………..72

3.4.2 The transcript levels of Zm *BOR1* correlate with the difference in B deficiency in maize……………………………...………………………………75

3.4.3 The expression levels of Os *BOR1* gene correlates with the ability in tolerance to B deficiency of rice……………………………………………………….77

Chapter 4: Analysis of *A. thaliana GLIP1* gene in relation to B deficiency…………………………………………………………79

4.1 Introduction……………………………………………………………….……79

4.2 Materials and methods………………………………………………………80

4.2.1 Plant materials…………………..…………………………………………80

4.2.2 Plant growth conditions……………………………………………………80

4.2.3 Quantification of transcript accumulation by real-time PCR……………...…81

4.2.4 Measurement of B and Ca concentration in plant tissues…………….……………82

4.3 Results……………………………………………………………...……………83

4.3.1 Identification of Arabidopsis *GLIP1* as a gene up-regulated under B limitation in roots……………………………………………………………………….83

4.3.2 Isolation of *GLIP1* T-DNA insertion mutants……………………………….86

4.3.3 Expression and functional analysis of *GLIP1*……………………….……....88

4.3.3.1 Phenotypic analysis of *GLIP1* T-DNA insertion mutants……………88

4.3.3.2 B concentration in *GLIP1* T-DNA insertion plants…………………..91

4.3.3.3 Phenotypic analysis of *GLIP1* T-DNA insertion mutants under various nutrient conditions...……….…...………………………………………93

4.3.3.4 *GLIP1* transcript accumulation in T-DNA insertion lines….……..…99

4.3.3.5 Ca concentration in *GLIP1* T-DNA insertion plants……….……..…101

4.4 Discussion…………………………………………………………………...…103

4.4.1 *GLIP1* is induced by B deficiency…………..…………………………………103

4.4.2 A possible role of *GLIP1* in plant growth under B or Ca deficiency……....103

CONCLUSION……………………………………….…………………106

REFERENCE…………………………….....………………………108

APPENDIX……………………………………………………...…121

ACKNOWLEDGEMENTS..128

List of Figures and Tables

Figure 1-1 Structure of RG-II..18

Figure 1-2 The model of B transport pathways and mechanisms in plants…..……..…22

Figure 2-1 Sequence alignment and phylogenetic analysis of BOR-like proteins in wheat, *A. thaliana* and rice…...…….……………………...……..………...36

Figure 2-2 B transport activity of Ta BOR1s in BY-2 cells…...…………………….…42

Figure 2-3 Subcellular localization of Ta BOR1s:GFP in *A. thaliana* leaf cell...……...44

Figure 2-4 Accumulation of Ta *BOR1* transcripts in roots and shoots in response to B conditions…………………………………………………………...………47

Figure 2-5 Model of the possible roles of Ta BOR1s in roots under normal, low (-B) and excess (++B) B conditions………………………………………………..51

Table 3-1 Genes and gene-specific primers used for the real-time PCR experiments…56

Figure 3-1 Transcript levels of wheat *BOR1*-like gene, Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* **in roots at the vegetative stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 μM B) or sufficient (10 μM B) condition…………………………………………………………………...58

Figure 3-2 Transcript levels of wheat *BOR1*-like gene, Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* **in leaves at the vegetative stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 μM B) or sufficient (10 μM B) condition…………...…………..…………………….…………………….……….59

Figure 3-3 Transcript levels of wheat *BOR1*-like gene, Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* **in roots at the reproductive stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition……………………..……………………………………………..60

Figure 3-4 Transcript levels of wheat *BOR1*-like gene, Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* **in leaves at the reproductive stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition………………………………………...…………..…………62

Figure 3-5 Transcript levels of wheat *BOR1*-like gene, Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* **in ear** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition….…………………………………………...……….…………63

Figure 3-6. **Summary of relative expression of wheat *BOR1*-like genes**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage, leaves at the reproductive stage and ear of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient condition…………….…..……………………………………64

Figure 3-7 Transcript levels of Zm *BOR1*-like gene **in roots and leaves at the vegetative stage and ear at the reproductive stage** of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficient (0 µM B) or sufficient (20 µM B) condition…………………………………….........………66

Figure 3-8 Transcript levels of Zm *BOR1*-like gene **in roots, leaves and baby corn at the reproductive stage** of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficient (0 µM B) or sufficient (20 µM B) condition…..……………..……………....…………………………….….….67

Figure 3-9 **Summary of relative expression of Zm *BOR1*** in roots and leaves at the vegetative stage, roots and leaves at the reproductive stage, ear and baby corn of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficient condition…………...……………………………………........………68

Figure 3-10 Transcript levels of **Os *BOR1* gene in roots and leaves at the vegetative stage, roots and leaves at the reproductive stage and panicle** of SPR1 (Suphanburi1; B-efficient genotype) and CNT1 (Chainart1; B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition….…………………………………………………………………70

Figure 3-11 **Summary of relative expression of Os *BOR1*** in roots and leaves at the vegetative stage, roots and leaves at the reproductive stage and panicle of SPR1 (Suphanburi1; B-efficient genotype) and CNT1 (Chainart1; B-inefficient genotype) under B deficient condition….….……………………………...…..71

Table 4-1 Genes and gene-specific primers used for homozygous T-DNA insertion line selection and nucleotide sequence determination……...…………………...……80

Figure 4-1 Relative mRNA levels of the 16 candidate genes that were up-regulated under B deficiency from microarray data were verified by using quantitative real-time PCR in roots of Col-0 plants……………………………………………..…84

Figure 4-2 Accumulation of *GLIP1* (At5g40990) mRNA in roots of Col-0 plants…..…………………………………………………………………….85

Figure 4-3 *GLIP1* mutant alleles…...……………………………..…………..…..…87

Figure 4-4 Root length of *GLIP1* T-DNA lines under 0.03, 0.1, 0.3 or 100 μM B at 10-14 days after germination……..…………..……………………………...……89

Figure 4-5 Root elongation of *GLIP1* T-DNA lines and Col-0 plants after germination 7, 10 or 14 days under 0.03 μM B…………………..……………………………90

Figure 4-6 The B concentration in roots (A) and shoots (B) of Col-0 and *GLIP1* T-DNA lines for *GLIP1*……………………………………………………………...92

Figure 4-7 Root length (A) and shoot fresh weight (B) of Col-0 and *GLIP1* T-DNA lines under nutrient deficient conditions after germination 14 days…………………...94

Figure 4-8 Root length (A) and shoot fresh weight (B) of Col-0 and *GLIP1* T-DNA lines under 0, 0.15, 0.20, 0.25 or 2 mM Ca conditions………………………………..95

Figure 4-9 Plants grown in solid medium containing 0, 0.15, 0.20, 0.25 or 2 mM Ca for 14 days……………………………………………….............................96

Figure 4-10 Accumulation of *GLIP1* mRNA in roots (A) and shoots (B) of Col-0 and *GLIP1* insertion lines and the wild-type plants…………………………………100

Figure 4-11 The Ca concentrations in roots (A) and shoots (B) of Col-0 and *GLIP1* T-DNA lines……..…………………………………………………………......102

Figure 4-12 Models of Ca transport (A) and B transport (B) in *A. thaliana* roots……105

List of abbreviations in this thesis

A. thaliana	*Arabidopsis thaliana*
B	boron
Ca	calcium
cDNA	complementary DNA
CTAB	cetyltrimethylammonium bromide
EST	expressed sequence tag
GFP	green fluorescent protein
IAA	indole acidic acid
ICP-MS	inductively coupled plasma-mass spectrometry
LB / RB	left border / right border
MIP	major intrinsic protein
NIP	nodulin 26-like intrinsic protein
PC	phosphatidylcholine
PCR	polymerase chain reaction
PVP	polyvinypyrrolidone
RG-II	rhamnogalacturonan-II
RG-II-B	borate rhamnogalacturonan II complex
RNA	Ribonucleic acid
RT	reverse transcription
T-DNA	transferred DNA

OUTLINE

This thesis consists of outline, four chapters and conclusion. The contents of the four chapters are as followers. Chapter 1 is the part of introduction about physical and chemical proportions of boron (B), physiological function of B, B transport system and B deficiency response in plants. Chapter 2 is characterization of the genes responsible for B transport under B deficiency in wheat. The Ta *BOR1* genes were cloned and their expression were investigated. Chapter 3 is examination of *BOR1*-like gene transcript accumulation in crops. *BOR1*-like gene expression patterns in response to B stress among two genotypes with different B efficiency of Thai wheat, maize and rice were determined. In Chapter 4, I have identified novel genes that response to B deficiency in *Arabidopsis thaliana,* and expression and functions of *GLIP1* was investigated.

CHAPTER 1

Introduction

Introduction consists of four sections. The first section describes the chemical properties and distribution of boron (B) in nature. The second section describes the physiological functions of B in plants including roles for B in cell walls, membrane function, and metabolic pathways. The third section describes the transport mechanism of B in plants. Finally, physiological response of plant to low B and B deficiency in wheat, maize and rice are described in the last section.

1.1 properties and distribution of B in nature

B is a member of the metalloid group of elements from Group IIIA of the periodic table, with an atomic number of 5 and a relative atomic mass of 10.811 g mol^{-1}. More than one hundred borate minerals contain B in oxidation state of +3. B contains characteristics between metals and non-metals (Naghii, 1999). Its chemical properties are very different from those of the other elements in this group, such as aluminium or gallium. Its unique properties make B an important element in organic chemistry. Crystalline B is chemically inert and resistant to abolish by boiling hydrofluoric or hydrochloric acid (Tanaka and Fujiwara, 2008). The rate of oxidation of B depends upon the crystallinity, particle size, purity and temperature. B appears to interact with other biological substance, such as polysaccharides, pyridoxine, riboflavin, dehydroascorbic acid, and the pyridine nucleotides (Devirian and Volpe, 2003).

B has two naturally occurring stable isotopes, ^{11}B (80.1%) and ^{10}B (19.9%) that are found in the form of borax ore or tincal ($Na_2B_4O_7x10H_2O$), boric acid (H_3BO_3), colemanite [$CaB_3O_4(OH)_3^{-}H_2O$], kernite or rasorite ($Na_2B_4O_7x4H_2O$), ulexite ($NaCaB_5O_9xH_2O$) and borates (salt or ester of boric acid) in the oceans, sedimentary rocks, coal, shale and some soils (IPCS, 1998; Smallwood, 1998). In plant and animal cells, B primarily exists (more than 98%) as boric acid (Tanaka and Fujiwara, 2008). Boric acid (H_3BO_3) is mostly present as non-charged at neutral pH and in this condition it exists as odourless, colorless, translucent crystals or white granules or powder at ambient temperatures (O'Neil et al., 2004). The molecular radius of boric acid is 2.573 Å. Boric acid is a small molecule with three valence electrons and a very weak Lewis acid with p*K*a of 9.42. Boric acid form complexes with a wide variety of sugars and other compounds containing *cis-hydroxyl* groups such as phenols, organic acids and some polymers (Marques-Alves, 2010).

B is widely distributed in nature, with an average concentrations of around 10 mg/kg in the Earth's crust (ranged from 5 mg/kg in basalts to 100 mg/kg in shales), around 4.5 mg/l in the ocean and 3-30 µg/l in the rivers (Power and Woods, 1997; Marques-Alves, 2010). Plant tissues found to contain 10-100 mg/kg on dry weight basis (Shaaban, 2010). When B was applied to soil, 10% of B was absorbed by plants, 30-40% of B left in the soil, and 40-60% was leached out from the soil (Eguchi and Yamanda, 1997). Under soil pH conditions (pH 5.5-7.5), B is mainly present in a non-ionized form in soil solution. It may be the main reason why B can be leached so easily from the soil (Chaudhary et al., 2005).

1.2 The physiological role of B in plant

B is known as an essential element for all vascular plants since 1923. Warington observed that plants required a continuous supply of B. This is an important point for understanding of B function in plant growth until now (Devirian and Volpe, 2003; Blevins and Lukaszewski, 1998). The major roles of B are classified and described as follows: a structural role in cell walls; in membrane function; and in metabolic pathways (Brown et al., 2002).

1.2.1 Role of B in cell wall structure

The primary cell wall of higher plants is an important factor determining cell size and shape during plant development (Blevins and Lukaszewski, 1998). The mechanical properties of growing cell wall can be modified by crosslinks between their major components, cellulosic polymers, and matrix polymers such as hemicellulosic and pectic polysaccharides (Blevins and Lukaszewski, 1998). The close relationship between the primary cell walls and B nutrition has been observed for many years. Most of the cellular B (> 90%) has been localized in the cell wall fraction where B is associated with pectinacious compounds.

Borate-rhamnogalacturonan-II (RG-II-B) complex was found in cell walls of many plant species such as *Brassicaceae*, *Cucurbitaceae*, *Leguminosae*, *Apiaceae*, *Chenopodiaceae*, *Solanaceae*, *Asteraceae*, *Liliaceae*, *Araceae*, *Amaryllidaceae* and *Gramineae* (Matoh et al., 1996 ; Blevins and Lukaszewski, 1998). The borate ester was located on C-2 and C-3 of two of the four 3'-linked apiosyl residues of dimeric RG-II. The formation of this complex is essential for cell wall structure and function (Figure 1-

1) (O'Neill et al., 2004; Camacho-Cristobal et al., 2011). Furthermore, Ryden et al. (2003) suggested that RG-II-B complex plays a role in the expanded primary wall and in secondary wall structure or assembly. B and RG-II may also interact in processes beyond more cell-wall cross linking (Goldbach and Wimmer, 2007). *In vivo* cross-linking of apiose residues by B in the pectic polysaccharide RG-II has been convincingly demonstrated, supporting earlier suggestions that the primary function of B in plants is a structural role, relating to the stability of the cell wall (Warington, 1923; O'Neill et al., 2001 and 2004).

1.2.2 Role of B in membrane function

In plants, B is localized in membranes (Tanada, 1983; Bonilla et al., 2010). B plays a role in maintaining the structural integrity and physiological functions of plasma membrane (Yang and Li, 1999; Blevins and Lukaszewski, 1994). B seems to perform its role by forming complex with membrane compounds containing *cis*-diol groups, such as glycoproteins and glycolipids (Yang and Li, 1999), which are structural constituents of the plasma membrane (Marques-Alves, 2010; Goldbach and Wimmer, 2007). The binding of B to these compounds may change the conformation of proteins and lipids, and lead to the alternation of their functions (Yang and Li, 1999).

It is also possible that B affect the activity of ion channels in the plasma membrane (Yang and Li, 1999). The quantities of B in membranes were not large compared with those in cell wall function, however, they were important for ion uptake (Blevins and Lukaszewski, 1998). In membranes, B could be involved in ion transporters and redox reactions by stimulating enzymes like nicrotinamide adenine

dinucleotide (NADH) oxidase (Blevins and Lukaszewski, 1994). Parr and Loughman (Blevins and Lukaszewski, 1994) found that B increased membrane transport of chlorine (Cl) and phosphorus (P). These results are explained as a B stimulation of the plasmalemma ATPase. Schon et al. (1990) found a hyperpolarization of membranes of sunflower roots within 3 min of B addition to a B free medium, whereas an instantaneous stimulation of the plasma membrane NADH oxidase was reported after B addition to low B carrot cell cultured (Bonilla et al., 2010). Other studies have shown that supplemental B stimulates proton pumping in plants, causes hyperpolarization of the membrane potential, and increase K^+ uptake (Blevins and Lukaszewski, 1994). It has also been reported that B deficiency decrease net nitrate uptake by repressing expression of root plasma membrane H^+-ATPase gene (Gonzalez-Fontes et al., 2008).

1.2.3 Role of B in metabolic pathways

Primary cell wall structure and membrane functions are closely related to B element, while, role of B in plant metabolism is still a considerable discussion. On the basis of the stability of known borate monoesters in aqueous systems and the distribution of *cis*-diol-binding sites in cells, it seems likely that B monoesters play a relevant metabolic function (Goldbach and Wimmer, 2007). The mechanisms by which it binds to enzymes or to cofactors are not presently known and must be investigated further. One hypothesis is that B alters metabolic reactions by binding to substrate compounds (Devirian and Volpe, 2003). Many physiological impairments caused by B deficiency have been reported and it has been postulated that B may be involved in a wide number of metabolic pathways (Brown et al., 2002). The possible roles of B in

metabolic pathways include phenol metabolism, nitrogen metabolism, ascorbate metabolism, indole acetic acid (IAA) metabolism, sugar transport, cell wall synthesis, lignification, cell wall structure integrity, carbohydrate metabolism, ribose nucleic acid (RNA) metabolism, respiration, and etc. (Ahmad et al., 2012).

For instances, B is one of the nutrients responsible for the changes in concentration and metabolism of phenolic compounds in vascular plants (Herrera-Rodriguez et al., 2010). Accumulation of phenolic takes places under B deprivation (Brown et al., 2002; Herrera-Rodriguez et al., 2010). Camacho-Cristobal et al. (2002) demonstrated that the concentration of phenylpropanoid as well as the polyphenol oxidase and phenylalanine ammonia-lyase activities increased mainly in tobacco leaves during short-term B deficiency. The involvement of B on phenol metabolism may also be a secondary consequence of disruptions in carbon metabolism as a result of a role for B as a structural element in the cell wall or cell membrane (Brown et al., 2002). B has also been implicated in nitrogen metabolism. A decreased nitrate uptake was observed in B-deficient tobacco plants. It is probably due to a lower expression of plasma membrane H^+-ATPase (Brown et al., 2002; Camacho-Cristobal and Gonzalez-Fontes, 2008). There is substantial evidence supporting the association of B with ascorbate metabolism (Brown et al., 2002). Chandler and Miller (1946) and Blevins and Lukaszewski (1998) found that rutabaga treated with B had more ascorbate than untreated controls, and that following dehydration and storage, the B-treated plants maintained around twice as much ascorbate. Another function of B is auxin metabolism. B may also suppress IAA oxidase activity (Marschner, 1995). Bohnsack and Albert

(1997) demonstrated a 20-fold increase in IAA oxidation rate in root apices 24 h after B was withheld from the nutrient medium.

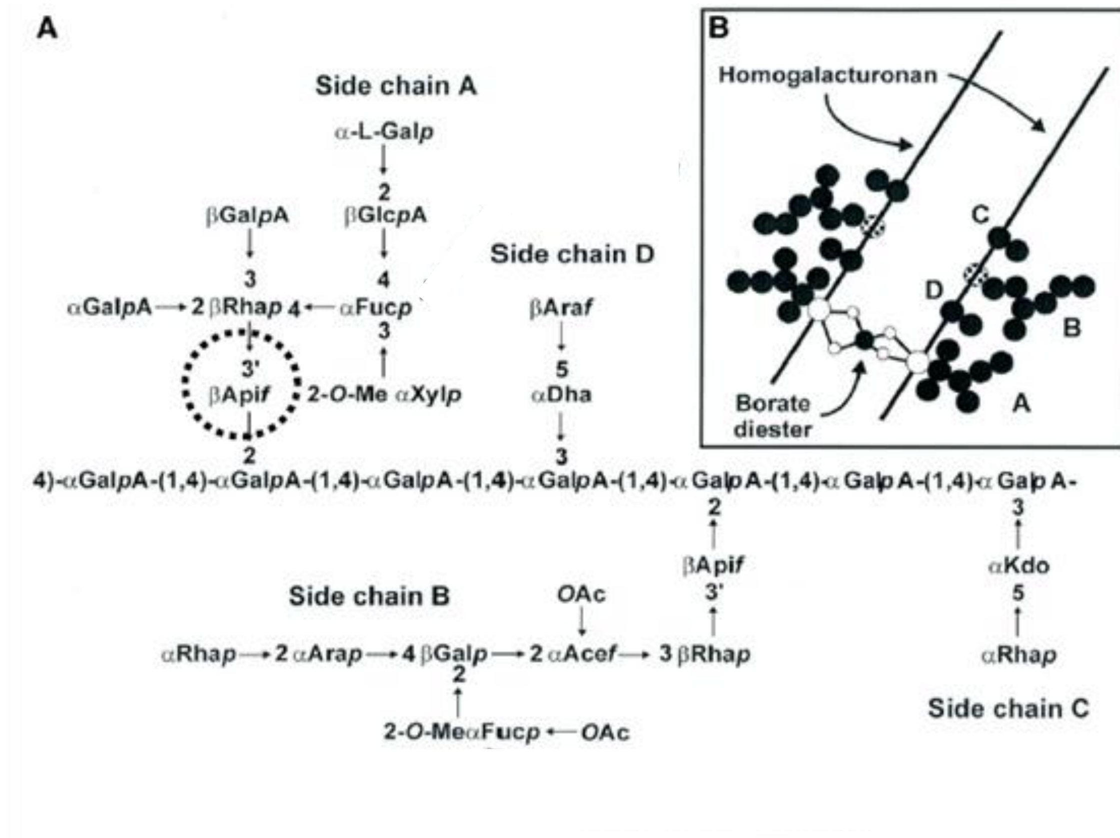

Figure 1-1. Structure of RG-II: A: shows the detailed structure of the RG-II monomer; the dotted circle indicates the apiose moiety responsible for cross-linking with borate, whereas the apiose at side chain B is not involved in the dimer formation. B: depicts the RG-II-B dimmer. Reproduced from Goldbach and Wimmer (2007)

1.3 B uptake and transport in plant under B limitation

B uptake is considered to be a passive process. In soils, B mostly is present as boric acid (H_3BO_3). This form of B is mobile and easily lost by leaching, but it can be taken up by plant roots (Nable et al., 1997). After taken up by roots, B is transported to the shoots via the xylem. The long distance translocation of solutes in the xylem depends on transpiration, and B is transported along the transpiration systems (Tanaka and Fujiwara, 2008; Landi et al., 2012). Physiological studies have revealed the presence of channel-mediated facilitated diffusion and energy-dependent active transport against concentration gradients in B transport systems. Two types of B transporters were identified in *A. thaliana*, NIP5;1 and BOR1, both of which are important for efficient uptake and transport of B across the plasma membrane under B limitation (Miwa and Fujiwara, 2010).

1.3.1 B uptake by root plants

B is absorbed by roots as undissociated boric acid (H_3BO_3). B is the only element that is taken up from the soil by plants not as an ion, but as an uncharged molecule (Marschner, 1995; Miwa and Fujiwara, 2010; Ahmad et al., 2012). B absorption by plant roots is closely related to pH and B concentration in the soil solution, and is probably a passive process (Brown and Hu, 1998). The factors affecting B uptake include soil type (texture, alkalinity, pH, organic matter content), B concentration, moisture, and plant species (Ahmad et al., 2012).

Under high B supply, B is absorbed by root through a passive diffusion across lipid bilayer (Tanaka and Fujiwara, 2008; Camacho-Cristobal et al., 2011). In fact, boric

acid has a very high permeability coefficient for lipid bilayer. This affirmation was supported by experimental data from lipid permeability coefficients calculated in both the membrane vesicles isolated from *Cucurbita pepo* and giant internodal cells of the *Chara corallina* (Camacho-Cristobal et al., 2011; Brown et al., 2002).

Several reports suggest that B uptake is facilitated through the major intrinsic proteins (MIPs), channel proteins. Takano et al. (2006) found nodulin 26-like intrinsic protein (NIP) 5;1 as a boric acid channel required for boric acid uptake and normal growth in *A. thaliana* under low B condition. NIPs belong to a subgroup of plant MIPs that function as channels for water and small uncharged molecules (Maurel, 2007). *NIP5;1* transcript accumulation is up-regulated more than 10-folds by low B condition and localized in the plasma membrane of root epidermal, cortical, and endodermal cells. Expression of *NIP5;1* facilitates the influx of boric acid to *Xenopus laevis* oocytes. The *NIP5;1* mutant line exhibited reduced B uptake by roots and growth defects under B limitation. These results suggested that NIP5;1 is a major plasma membrane boric acid channel required for efficient B import into *A. thaliana* roots under B limitation (Takano et al., 2006; Miwa and Fujiwara, 2010). Beside *A. thaliana*, Os *NIP3;1*, the closet homolog to At *NIP5;1*, has been also identified as a boric channel required for efficient growth under B limitation in rice (Hanaoka and Fujiwara, 2007). Expression of maize *PIP1* in *Xenopus oocytes* increases the boric acid uptake around 30% (Dordas et al., 2000).

1.3.2 B transport in plants

B is absorbed by root cells, and it is transported towards shoots. BOR1 is involved in xylem loading and is regulated for B transport from roots to shoots under B limitation (Dannel et al., 2002). BOR1 is an energy dependent high affinity transporter. *BOR1* was identified by positional cloning as the first B efflux transporter required for normal shoot growth of *A. thaliana* under low B condition (Takano et al., 2002). *BOR1* belongs to the bicarbonate transporter superfamily (SLC4) (Frommer and Wiren, 2002). It is localized to plasma membrane and *BOR1* decreased cellular B concentration when expressed in yeast, supporting that BOR1 plays a role as a B exporter (Takano et al., 2002 and 2008). BOR1 promoter-GFP fusions showed the expression of BOR1 in pericycle cells of the root stele. The activity of the BOR1 transporter was high under B limitation and rapidly down-regulated after exposure to high B condition (Takano et al., 2008). The *bor1-1* mutant of *A. thaliana* exhibited severe shoot growth inhibition and reduced fertility under low B condition (Noguchi et al., 1997). Taken together, it was established that BOR1 functions as xylem loading of B under low B condition by exporting B from stelar cells into the xylem (Figure 1-2) (Tanaka and Fujiwara, 2008; Takano et al. 2010). It has also been demonstrated that BOR1 proteins accumulate to a high level in response to low B condition in *A. thaliana* (Takano et al., 2005).

In addition to *BOR1*, it is also demonstrated that *NIP6;1* is important for B distribution in shoots (Tanaka et al., 2008). Amino acid sequence of NIP6;1 is the most similar to NIP5;1 among all NIP genes in the *A. thaliana* genome. NIP6;1 is also localized in the plasma membrane and facilitates boric acid uptake into oocytes (Tanaka et al., 2008). The *NIP6;1* mutant lines exhibited defects in the expansion of young

leaves. The B concentrations in young leaves of the mutant were lower than that of wild type plants under low B condition. *NIP6;1* promoter activity was observed in the phloem region at nodes of the stem. Taken together, these results suggest that NIP6;1 functions in xylem-phloem transfer for preferential distribution of B into young growing tissues (Tanaka et al., 2008; Miwa and Fujiwara, 2010).

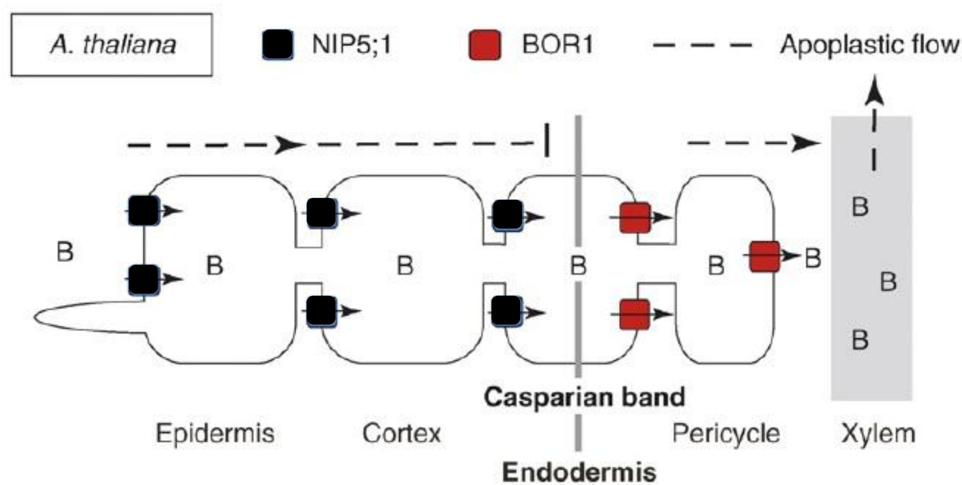

Figure 1-2. The model of B transport pathways and mechanisms in plants. In *A. thaliana* roots, under low B conditions, NIP5;1 imports boric acid into epidermal, cortical and endodermal cells, and BOR1 exports boric acid/borate from stelar cells (xylem loading). The endodermal Casparian band prevents the apoplastic flow of B into and back from the stele (primary vascular tissue that consists of pericyle, xylem and phloem). In the epidermis and cortex, NIP5;1 localized to the plasma membrane of the distal side, and in the endodermis, BOR1 localized to the plasma membrane of the proximal side. Redrawn based on Takano et al. (2008)

1.4 B deficiency response in plants

B deficiency is a major agricultural problem that affects vegetative and reproductive growth of plants resulting in the inhibition of cell expansion, the death of meristem and reduced fertility. The symptoms of B deficiency vary among crop species, but generally occur in the growing points or flower and fruiting parts of the plant. It is characterized by abnormal or retarded elongation of apical meristems (Benton, 2003).

1.4.1 Physiological response of plant to low B

B is essential for normal growth of plants because it promotes proper cell division and cell elongation in meristematic tissues and floral organs, cell wall strength, flowering pollination, pollen tube germination and elongation, seed set, and sugar translocation (Marschner, 1995).

B deficiency is a widespread problem for field crop production, and causes large losses of yield occur annually both quantitatively and qualitatively (Marschner, 1995; Shorrocks, 1997; Goldbach and Wimmer, 2007). B deficiency has been reported in more than 80 countries and for 132 crops over the last 60 years (Shorrocks, 1997; Ahmad et al., 2012). B deficiency has been commonly found in soils which are highly leached and developed from calcareous, alluvial and loessial deposits. There are several factors and conditions that affect soil deficient in B, including low soil organic matter content, sandy texture, high pH, liming, drought, intensive cultivation and more nutrient uptake than application, and the low available using fertilizers in micronutrients. (Ahmad et al., 2012).

B deficiency have been reported for many plant species and cultivars such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), cotton (*Gossypiumhirsutum* L.), soybean (*Glycine max* L.), oilseed rape (*Brassica napus*), alfafa (*Medicago sativa* L.), oil palm (*Elaeis guineensis*) (Dregne and Powers, 1942; Ferwerda, 1954; Brown and Ambler, 1969; Cheng and Rerkasem et al., 1993; Wei et al., 1998; Rerkasem and Jamjod, 2004; Rashid et al., 2009; Lordkaew et al.,2011; Savic et al., 2011). It is also known that B deficiency causes different effects on root elongation, IAA oxidase activity, sugar translocation, carbohydrate metabolism, nucleic acid synthesis, and pollen tube growth (Camacho-Cristobal et al., 2011).

1.4.2 B deficiency in wheat

Wheat (*Triticum aestivum* L.) is the major cereal crops grown in almost every part of the world. B deficiency in wheat has been reported included Bangladesh, Brazil, Bulgaria, China, Finland, India, Madagascar, Nepal, Pakistan, South Africa, Sweden, Tanzania, Thailand, USA, USSR, Yugoslavia, and Zambia (Shorrocks, 1997; Rerkasem and Jamjod, 2004). Its deficiency causes grain set failure and considerable yield losses in the wheat belt of the world's wheat growing countries (Rerkasem and jamjod, 2004; Ahmad et al., 2012).

For wheat, B requirement for reproductive development is higher than that for vegetative growth (Ahmad et al., 2012). Vegetative development of wheat is relatively insensitive to B deficiency, but lack of B during reproductive development can cause devastating yield loss through sterility (Subedi et al., 1998). Several previous studies reported that B deficiency was induced in vegetative growth in wheat only after B in the

nutrient solution had been depleted by plant uptake. The first symptom of B deficiency in field grown wheat is observed during anthesis. The florets remain open longer than normal. The effect of B deficiency has also been shown to cause poor anther and pollen development and the fertilization process (Huang et al., 2000). *In vitro* germination tests also showed that B was required for pollen germination and tube growth in wheat (Cheng and Rekasem, 1993).

There are several reports for genetic variation in B efficiency among wheat genotypes. A large variation among wheat genotypes has been observed in the response to low B condition. Wheat genotypes were classified based on B efficiency, namely, efficient, moderately efficient, moderately inefficient and inefficient (Rerkasem and Jamjod, 1997a). B-inefficient genotype were completely sterile and set no or just a few grains, while B-efficient genotypes set grain normally in low B condition in quartz river sand experiments. (Rerkasem and Jamjod, 1997a; Jamjod et al., 2004). However, the mechanisms of cultivar differences in wheat are still unknown (Nachiangmai et al., 2004).

1.4.3 B deficiency in rice

Rice (*Oryza sativa* L.) is one of the most important cereal crops and is a model species for monocotyledonous plants with a rather small genome. B deficiency has a serious impact on rice productivity. In rice, B deficiency results in white and rolled tips of emerging leaves, reduced plant height. Moreover, severe deficiency can cause the death of growing point although new tillers continue to be produced. In addition, B

deficiency at the panicle formation stage causes reduced-production of panicles (Dobberman and Fairhurst, 2000; Saleem et al., 2011).

B is also necessary in improving kernel quality of rice. Starch contents, grain size, shape and strength are increased by applying B at suitable time. Dunn et al. (2005) and Shaaban (2010) found that rice yields were greatest when soil B levels were between 0.25 to 0.33 ppm. Application of B to soil result in significantly greater yields than foliar B application and no B application. Positive responses to B application were observed in rice cultivars Basmti-370 and IR-6, a major cultivar in Punjab (Chaudhry et al., 1976). Similarly, paddy yield increased by 14-25% over control in cvs. Supper Basmati, Basmati-385 and KS-282 grown in rice belt of Punjab and in cv. IR-6 grown in Sindh province (Rashid et al., 2004). Yield increases are caused by the decrease (on lower portion of the ear) and increased productive tillers per hill. Lordkaew et al. (2013) indicated that B deficiency can cause male sterility and depressed pollen viability and grain set in rice.

1.4.4 B deficiency in maize

Maize (*Zea mays* L.) belongs to Graminae family. It is the most important crop among all cereal grain crops, such as wheat and rice. Maize is widely grown throughout the world in subtropical and temperate agroclimatic regions (Fageria et al., 1991; Martin et al., 1976). Most of the maize-growing areas are unsuitable condition i.e. infertile soil. Maize yield is low when plants are grown in infertile soils with low B (Sherrocks, 1997). Eltinge (1936) demonstrated that the visible effect of a lack of B in maize appeared on the seventh day in the form of a chlorosis of the tissue between the veins of the older

leaves, and the failure of the youngest leaves to unroll normally. The symptom of B deficiency in maize is including thin and death anthers at pollen shedding time. B deficiency depressed commercial maize yield primarily through grain set failure. Maize requires a supply of available B, especially during tasseling and silking. The emergence of tassel and silk is suppressed and delayed under low B condition. The most common B deficiency symptom is missing kernels which results significantly decrease in yields (Eltinge, 1936; Lordkaew et al., 2011; Palta and Karadavut, 2011). For an open pollinated crop with separated female and male inflorescences like maize, understanding the molecular basis of B deficiency response is useful for the management of B deficiency through its sensitive analysis. This understanding may also benefit the production of hybrid maize seed, with the primary concern being successful pollination and fertilization (Lordkaew et al., 2011).

CHAPTER 2

Differential Expression of Three *BOR1* Genes Corresponding to Different Genomes in Response to B Conditions in Hexaploid Wheat (*Triticum aestivum* L.)

2.1 Introduction

Wheat (*Triticum aestivum* L.) is a staple cereal throughout the world (Rashid et al., 2011). B deficiency is a critical problem for wheat production in many countries and regions with high rainfall including many areas of the subtropics (Rerkasem and Jamjod, 2004; Emon et al., 2010). Wheat is more susceptible to B deficiency than rice, maize and some dicotyledons including soybean and mungbean. In wheat, B deficiency depresses yield primarily through grain set failure (Rerkasem and Jamjod, 2004). To overcome the problem of B deficiency in wheat, it is of fundamental importance to understand the molecular mechanisms of B transport through identification of transporters. In case of *A. thaliana*, it is reported that high expression of *BOR1* leads to tolerance to B deficiency (Miwa et al., 2006). Besides *A. thaliana*, *BOR1*-like genes widely present in eukaryotic genomes. So far, functional *BOR1*-like genes have been identified from *Saccharomyces cerevisiae* (Takano et al., 2002), Eucalyptus (Domingues et al., 2005), rice (Nakagawa et al., 2007), *Brassica napus* (Sun et al., 2012) and grapes (Perez-Castro et al., 2012). To date, *BOR1* genes in wheat have not yet been characterized.

In the present study, I studied expression of three orthologs of Os *BOR1*, Ta *BOR1.1* to *1.3*, from wheat. Ta BOR1s were identified by Dr. T. Fujibe that these

encode functional efflux type of B transporters. I demonstrated that mRNA accumulation of Ta *BOR1s* was regulated by B status, which is different from those of At *BOR1* and Os *BOR1*.

2.2 Materials and Methods

2.2.1 Plant materials and cultures

Wheat (*Triticum aestivum* L.) seeds were germinated on 0.3 mM $CaCl_2$ plate for 3 d at 4°C after surface sterilization in 1% H_2O_2 for 60 min and then incubated in a vertical position for 3 d at 20 to 25°C under fluorescent lamps in a long-day condition (16 h light/8 h dark cycle). The seedlings were transplanted into 1.5 L pot wrapped with black plastic tape and containing half-strength Hoagland nutrient solution supplemented with 18 nM, 18 μM or 1 mM boric acid. Wheat seedlings were grown in a growth chamber under controlled conditions (temperature 20 to 25°C; photoperiod 16 h light/8 h dark; relative humidity 60-70%).

2.2.2 Sequence alignment and phylogenetic analysis

A similarity search of the wheat EST was performed with the Os BOR1 amino acid and cDNA sequence using the BLAST programs in Wheat Genetic Resources Database (KOMUGI) at National BioResource Project website (NBRP; http://www.shigen.nig.ac.jp/wheat/komugi). The amino acid sequences of all putative wheat BOR1s were aligned by the ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2). A phylogenetic tree, based on BOR family members from *A. thaliana*, rice and wheat, was constructed using Phylodendron software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). Aligned sequences were used to generate the phylogenetic tree (Figure 2-1A).

2.2.3 Plasmid constructions (by Dr. Takahiro Fujibe)

The Ta BOR1s-GFP fusion was constructed by Dr. T. Fujibe as follows. Total RNA was extracted from shoots using an RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the PrimeScript RT reagent kit (Takara) with oligo(dT)$_{16}$ primer. The Ta *BOR1.1* to *1.3* cDNA was amplified by PCR using primers 5´-ATACGCTCTCCGATTCCTTC-3´ and 5´-ACCGTGCATAAGTAGACATC-3´ for Ta *BOR1.1*; 5´-AAAAGCGTAATCTGGG TCTCT-3´ and 5´-ACCGTGCATAAGTAGACATC-3´ for Ta *BOR1.2*; 5´-GGTGAAT CCCTTGTCGGGTT-3´ and 5´-ACCGTGCATAAGTAGACATC-3´ for Ta *BOR1.3*. The amplified fragments were cloned into the ZeroBlunt vector (invitrogen) according to the manufacturer's instructions. After subcloning, the nucleotide sequences of the Ta *BOR1.1* to *1.3* cDNA were confirmed by DNA sequencing. The *Eco*RI fragments from the plasmids containing Ta *BOR1.1* to *1.3* were subcloned into the *Eco*RI vector fragment from the binary vector pYES2 (Invitrogen). The *Kpn*I-*Nco*I fragments from the plasmids containing Ta *BOR1.1* to *1.3* were then subcloned into *Kpn*I-*Nco*I fragment from the vector pTF521, which is the pYES2 vector containing GFP. The resulting plasmids contained Ta *BOR1.1* to *1.3*-GFP. For the transient expression, Maize *ubiquitin1 promoter* (*pUbi*) was amplified from pANDA vector (Miki and Shimamoto, 2004) by PCR using primers, 5´-ATGAAGCTTGCAGCGTGACCCGGTC GTGC-3´ and 5´-CATGTTAACCATCTGCAGAAGTAACACCAAAC-3´. *Hin*dIII and *Hpa*I (blunt-end) recognition sites are underlined, respectively. The *Hin*dIII-*Hpa*I fragment of *pUbi* was subcloned into the *Hin*dIII-*Sal*I (*Sal*I was blunt-ended) site of the binary vector pMDC32 (Curtis and Grossniklaus, 2003). The Ta *BOR1.1* to *1.3-GFP*s

were amplified using primers, 5´-ATGCTGCAGATGGAGGAGAGCTTCGTGCC-3´ and 5´-TTACTTGTACAGCTCGTCCA-3´. *Pst*I recognition site is underlined. The PCR products containing Ta *BOR1.1* to *1.3*:*GFPs* were then subcloned into *Pst*I-*Hpa*I fragment from the binary vector pMDC32 containing *pUbi*. The resulting plasmids contained p*Ubi*::Ta *BOR1.1* to *1.3:GFPs* were used to determine the Ta BOR1s activities in BY-2 cells and the subcellular localization of Ta BOR1s in *A. thaliana* leaf cell.

2.2.4 Determination of Ta BOR1 activity in BY-2 cells (by Dr. Takahiro Fujibe)

Transformation of BY-2 cells was performed by Dr. T. Fujibe as follows. 5 ml of 72 h-old BY-2 culture was co-cultivated with 100 µl of *Agrobacterium* EHA101 carrying plasmids containing *pUbi*::Ta *BOR1.1* to *1.3:GFP* and incubates for 42 to 60 h at 27°C dark. The cells were incubated with medium containing 1 mM boric acid for 60 min. The transformed BY-2 cells were mixtures of a number of independently transformed cells. The soluble fraction of BY-2 cells was sampled, and the B concentrations were determined using ICP-MS as described by Takano et al. (2002).

2.2.5 Subcellular localization of Ta BOR1s-GFP (by Mr. Sheliang Wang)

The subcellular localization of Ta BOR1s was investigated by Mr. S. Wang. For transient expression of Ta BORs-GFP in *A. thaliana* leaf cells, the method was followed from Grefen et al. (2010) with following modifications. For co-cultivation of *Agrobacterium* strain GV3101:pMP90 with *A. thaliana* seedlings, MGRL medium (Takano et al., 2005) containing 10 µM boric acid instead of MS basal salts medium

was used. After the co-cultivation for 24 h, the seedlings were washed two times with 0.003 % NaClO, then two times with sterile water, and cultured in fresh MGRL medium containing 10 µM boric acid for 24 h.

The construct for *pUBQ10::mCitrine-NIP5;1* (Alassimone et al., 2010) was provided by N. Geldner. The cotyledons were incubated with 10 µM FM4-64 for at least 5 min before the observation. Confocal images were taken with a confocal laser scanning microscope (Leica TCS-SP8) equipped with a water-immersed x 40 lens with the excitation wavelength 488 nm and following detection wavelengths, respectively: 500–540 nm for GFP; 510-600 nm for mCitrine; and >650 nm for FM4-64.

2.2.6 RNA extraction and quantitative real-time PCR

Total RNA was extracted from roots and shoots and the RNA was then reverse-transcribed to cDNA as described above. The absolute real-time PCR amplification was performed using a Thermal Cycler Dice (Takara) with SYBR Premix Ex Taq II (Takara). The primers used in real-time PCR were designed using Primer3 software (http://frodo.wi.mit.edu/primer3). The sequences of the primers were as follows: 5´-CCGGTATCAGGATCCTAGCG-3´ and 5´'-ATGCCAATGTCTGCACCGCG-3' for Ta *BOR1.1*; 5´-GCGGCTCACCTGCTACAAGC-3´ and 5´-ATGCCAATGTCTGCAC TGCC-3´ for Ta *BOR1.2*; and 5´-ACATCTTCTTCGCCTCCGCG-3´ and 5´-ATGCCA ATGTCTGCACTGCT-3´ for Ta *BOR1.3.*

Eight-fold serial dilution series of the plasmid carried *BOR1-GFP* fusion genes as described above, ranging from $1x10^{-3}$ to $1x10^{4}$ copies/µl, were used to construct the standard curves for Ta *BOR1.1*, Ta *BOR1.2* and Ta *BOR1.3.* The concentration of the

plasmid was estimated by OD_{260} and the number of copies/µl of standards was calculated according to the following formula (Yin et al., 2001):

$$\text{Copies/ml} = \frac{6.023 \times 10^{23} \times C \times OD_{260}}{MWt}$$

Where C= 5×10^{-5} g/ml for DNA and MWt= molecular weight of PCR product (base pairs x 6.58×10^{2} g)

2.3 Results

2.3.1 Phylogenetic analysis of Ta BOR1s

A database search of the wheat genome for Os *BOR1*-like genes identified three genes. Based on the predicted amino acid sequences, I named these genes, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3.* To confirm the nucleotide sequences of the Ta BOR1s in the database, I amplified the corresponding cDNA from mRNA isolated from leaves of wheat cultivar Chinese Spring by reverse transcription mediated PCR. Among the Ta BOR1, Ta BOR1.1 showed 98% and 99% identity at the predicted amino acid sequences with Ta BOR1.2 and Ta BOR1.3, respectively. The predicted amino acid sequence of Ta BOR1.2 showed 99% identity with Ta BOR1.3. All three Ta BOR1 genes are 91% identical in amino acid sequence to Os BOR1 and showed 79-80% similarity on the amino acid level to At BOR1 (Figure 2-1A). The phylogenetic analysis showed that Ta BOR1.1, Ta BOR1.2 and Ta BOR1.3 are very similar each other and these genes are clustered together with Os BOR1 and At BOR1, 2, 3 (Figure 2-1B).

(A)

```
Ta BOR1.2     MEESFVP-----LRGIKNDVRGRLTCYKQDWTGGFSAGIRILAPTTYIFFASAIPVISFG 55
Ta BOR1.3     MEESFVP-----LRGIKNDVRGRLACYKQDWTGGFSAGIRILAPTTYIFFASAIPVISFG 55
Ta BOR1.1     MEESFVP-----LRGIKNDVRGRLACYKQDWTGGFSAGIRILAPTTYIFFASAIPVISFG 55
Os BOR1       MEESFVP-----LRGIKNDLHGRLQCYKQDWTGGFRAGIRILAPTTYIFFASAIPVISFG 55
At BOR1       MEETFVP-----FEGIKNDLKGRLMCYKQDWTGGFKAGFRILAPTTYIFFASAIPVISFG 55
At BOR4       MEEERVDSSKRLFRGIVADLRGRALCYKEDWVAGLRSGFGILAPTTYIFFASALPVIAFG 60
              ***  *      :.**  *::**  ***:**..*: :*: *************:***:**

Ta BOR1.2     EQLERSTDGVLTAVQTLASTALCGIIHSIVGGQPLLILGVAEPTVIMYTFMFSFAKDRAD 115
Ta BOR1.3     EQLERSTDGVLTAVQTLASTALCGIIHSIVGGQPLLILGVAEPTVIMYTFMFSFAKDRAD 115
Ta BOR1.1     EQLERSTDGVLTAVQTLASTALCGIIHSIVGGQPLLILGVAEPTVIMYTFMFSFAKDRAD 115
Os BOR1       EQLERNTDGVLTAVQTLASTALCGIIHSFLGGQPLLILGVAEPTVLMYTFMFNFAKDRPD 115
At BOR1       EQLERSTDGVLTAVQTLASTAICGMIHSIIGGQPLLILGVAEPTVIMYTFMFNFAKARPE 115
At BOR4       EQLSRDTEGALSTVETLASTALCGVIHSILGGQPLLILGVAEPTVLMYVYLYNFAIGRPE 120
              ***.*.*:*.*::*:******:**:***::***************:**.:::.**  *.:

Ta BOR1.2     LGPNLFLAWAGWVCVWTALLLFLLAVLGACSIINRFTRIAGELFGLLIAMLFMQQAIKGL 175
Ta BOR1.3     LGPNLFLAWAGWVCVWTALLLFLLAVLGACSIINRFTRIAGELFGLLIAMLFMQQAIKGL 175
Ta BOR1.1     LGPNLFLAWAGWVCVWTALLLFLLAVLGACSIINRFTRIAGELFGLLIAMLFMQQAIKGL 175
Os BOR1       LGRRLFLAWTGWVCVWTAILLFLLAILGACSIINRFTRIAGELFGLLIAMLFMQQAIKGL 175
At BOR1       LGRDLFLAWSGWVCVWTALMLFVLAICGACSIINRFTRVAGELFGLLIAMLFMQQAIKGL 175
At BOR4       LGKQLYLAWAAWVCVWTALLLFVMAILNTADIINRFTRVAGELFGMLISVLFIQQAIKGM 180
              **  *:***:.*******::**::*: .:..*******:******:**::**:******:

Ta BOR1.2     VDEFRIPERENIKALQFVPSWRFANGMFAIVLSFGLLLTALRSRKARSWRYGAGWLRGFI 235
Ta BOR1.3     VDEFGVPERENIKALQFVPSWRFANGMFAIVLSFGLLLTALRSRKARSWRYGAGWLRGFI 235
Ta BOR1.1     VDEFRIPERENIKALQYIPSWRFANGMFAIVLSFGLLLTALRSRKARSWRYGAGWLRGFI 235
Os BOR1       VDEFRIPERENRKALEFVSSWRFANGMFAIVLSFGLLLTALRSRKARSWRYGTGWLRGFI 235
At BOR1       VDEFRIPERENQKLKEFLPSWRFANGMFALVLSFGLLLTGLRSRKARSWRYGTGWLRSLI 235
At BOR4       VSEFGMPKDEDSKLEKYKFEWLYTNGLLGLIFTFGLLYTALKSRKARSWRYGTGWYRSFI 240
              *.** :*: *: *  ::  .* ::**::.::::**** *.*:**********:** *.:*
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Ta BOR1.2    ADYGVPLMVLVWTGVSYIPHDSVPKGIPRRLFSPNPWSPGAYDNWTVIKDMLQVPVMYII 295

Ta BOR1.3    ADYGVPLMVLVWTGVSYIPHDSVPKGIPRRLFSPNPWSPGAYDNWTVIKDMLQVPVMYII 295

Ta BOR1.1    ADYGVPLMVLVWTGVSYIPHDSVPKGIPRRLFSPNPWSPGAYDNWTVIKDMAQVPVMYII 295

Os BOR1      ADYGVPLMVLVWTGVSYIPYGSVPKGIPRRLFSPNPWSPGAYDNWTVIRDMPNVPLLYII 295

At BOR1      ADYGVPLMVLVWTGVSYIPAGDVPKGIPRRLFSPNPWSPGAYGNWTVVKEMLDVPIVYII 295

At BOR4      ADYGVPLMVVVWTALSFSTPSKLPSGVPRRLFSPLPWDSPSLSHWTVIKDMGKVSPGYIF 300

             *********:***.:*: . ..:*.*:******* **.. : .:***:::* .*.  **:


Ta BOR1.2    GAFMPATMIAVLYYFDHSVASQLAQQAEFNLRKPPSFHYDLLLLGFLTLMCGLIGIPPSN 355

Ta BOR1.3    GAFMPATMIAVLYYFDHSVASQLAQQAEFNLRKPPSFHYDLLLLGFLTLMCGLIGIPPSN 355

Ta BOR1.1    GAFMPATMIAVLYYFDHSVASQLAQQAEFNLRKPPSFHYDLLLLGFLTLMCGLIGIPPSN 355

Os BOR1      GAFIPATMIAVLYYFDHSVASQLAQQKEFNLRKPPSFHYDLLLLGFLTLLCGLIGIPPAN 355

At BOR1      GAFIPASMIAVLYYFDHSVASQLAQQKEFNLRKPSSYHYDLLLLGFLTLMCGLLGVPPSN 355

At BOR4      AAFIPALMIAGLYFFDHSVASQLAQQKEFNLKKPSAYHYDILLLGFMTLICGLLGLPPSN 360

             .**:** *** **:************ ****:**.::***:*****:**:***:*:**:*


Ta BOR1.2    GVIPQSPMHTKSLATLKHQILRNRLVATARQSMRQNASLSQLYNNMQDAYHQIQTPLIHQ 415

Ta BOR1.3    GVIPQSPMHTKSLATLKHQILRNRLVATARQSMRQNASLSQLYNNMQDAYHQIQTPLIHQ 415

Ta BOR1.1    GVIPQSPMHTKSLATLKHQILRNRLVATARQSMRQNASLSQLYNNMQDAYHQIQTPLIHQ 415

Os BOR1      GVIPQSPMHTKSLATLKHQLLRNRLVATARQSMSQNASLSQLYGSMQEAYQQMQTPLIYQ 415

At BOR1      GVIPQSPMHTKSLATLKYQLLRNRLVATARRSIKTNASLGQLYDNMQEAYHHMQTPLVYQ 415

At BOR4      GVLPQSPMHTKSLAVLKRQLIRRKMVKTAKESIRKRETSSQVYENMQEVFIEMDKSPLAQ 420

             **:***********.** *::*.::* **:.*:  . : .*:* .**:.: .::.. : *


Ta BOR1.2    QQTVKGLNELKDSTVQLASSMGNFDAPVDETIFDIEKEIDDLLPMEVKEQRLSNFLQAVM 475

Ta BOR1.3    QQSVKGLNELKDSTVQLASSMGNFDAPVDETIFDIEKEIDDLLPMEVKEQRLSNFLQAVM 475

Ta BOR1.1    QQSVKGLNELKDSTVQLASSMGNFDAPVDETIFDIEKEIDDLLPMEVKEQRLSNFLQAVM 475

Os BOR1      QPSVKGLNELKDSTVQMASSMGNIDAPVDETVFDIEKEIDDLLPIEVKEQRLSNLLQATM 475

At BOR1      QP--QGLKELKESTIQATTFTGNLNAPVDETLFDIEKEIDDLLPVEVKEQRVSNLLQSTM 473

At BOR4      TDP-SVIIELQDLKEAVMKSNDEEREGDEESGFDPEKHLDAYLPVRVNEQRVSNLLQSLL 479

             . : **:: .    .  .:     :*: ** **.:*  **:.*:***:**:**: :
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Ta BOR1.2      VGGCVAAMPLLKKIPTAVLWGYFAFMAIESLPGNQFWERILLLFTAPSRRYKVLEEYHTT 535
Ta BOR1.3      VGGCVAAMPLLKKIPTAVLWGYFAFMAIESLPGNQFWERILLLFTAPSRRYKVLEEYHTT 535
Ta BOR1.1      VGGCVAAMPLLKKIPTAVLWGYFAFMAIESLPGNQFWERILLLFTAPSRRYKVLEEYHTT 535
Os BOR1        VGGCVAAMPLLKKIPTSVLWGYFAFMAIESLPGNQFWERILLLFTAPSRRYKVLEEYHTT 535
At BOR1        VGGCVAAMPILKMIPTSVLWGYFAFMAIESLPGNQFWERILLLFTAPSRRFKVLEDYHAT 533
At BOR4        VAGAVLAMPAIKLIPTSILWGYFAYMAIDSLPGNQFFERLTLLFVPTSRRFKVLEGAHAS 539
               *.*.* *** :* ***::******:***:*******:**: ***...***:****  *::

Ta BOR1.2      FVETVPFKTIAMFTLFQTTYLLVCFGITWIPIAGVLFPLMIMLLVPVRQYILPKLFKGAH 595
Ta BOR1.3      FVETVPFKTIAMFTLFQTTYLLVCFGITWIPIAGVLFPLMIMLLVPVRQYILPKLFKGAH 595
Ta BOR1.1      FVETVPFKTIAMFTLFQTTYLLVCFGITWIPIAGVLFPLMIMLLVPVRQYILPKLFKGAH 595
Os BOR1        FVETVPFKTIAMFTLFQTMYLLVCFGITWIPIAGVLFPLMIMLLVPVRQYILPKLFKGAH 595
At BOR1        FVETVPFKTIAMFTLFQTTYLLICFGLTWIPIAGVMFPLMIMFLIPVRQYLLPRFFKGAH 593
At BOR4        FVEKVPYKSMAAFTLLQIFYFGLCYGVTWIPVAGIMFPVPFFLLIAIRQYILPKLFNPAH 599
               ***.**:*::* ***:*  *: :*:*:****:**::**: :::*:.:***:**::*: **

Ta BOR1.2      LNDLDAAEYEESP---AIPFNLAAQDID-VALGRTQSAEILDDMVTRSRGEIKRLNSPKI 651
Ta BOR1.3      LNDLDAAEYEESP---AIPFNLAAQDID-VALGRTQSAEILDDMVTRSRGEIKRLNSPKI 651
Ta BOR1.1      LNDLDAAEYEESP---AIPFNLAAQDID-VALGRTQSAEILDDMVTRSRGEIKRLNSPKI 651
Os BOR1        LTDLDAAEYEESP---AIPF-IAAQDID-VALARTQSAEILDDIVTRSRGEIKRLNSPKI 650
At BOR1        LQDLDAAEYEEAP---ALPFNLAAETEIGSTTSYPGDLEILDEVMTRSRGEFRHTSSPKV 650
At BOR4        LRELDAAEYEEIPGTPRNPLELSFRSNDSKRGVQEGDAEILDELTT-SRGELK-VRTLNL 657
               * :******** *     *: :: .           . ****:: * ****::   : ::

Ta BOR1.2      TSSGGTPVTELKGIRSPCMSEKAYSPRVNELRH-ERSPLGGRDSPRMGEARPSKLGEGST 710
Ta BOR1.3      TSSGGTPVTELKGIRSPCISEKAYSPRVAELRH-ERSPLGGRDSPRTGEARPSKLGEGST 710
Ta BOR1.1      TSSGGTPVTELKGIRSPCISEKAYSPRVTELRH-ERSPLGGRDSPRTGEARTSKLGEGST 710
Os BOR1        TSSGGTPVAELKGIRSPCISERAYSPCITELRH-DRSPLGGRGSPRTGETRSSKLGEGST 709
At BOR1        TSSSSTPVNNRS-------LSQVFSPRVSGIRLGQMSPRVVGNSPKPASCGRSPLNQSSS 703
At BOR4        NEDKGN---------------QIYP----------------KEKVKAGDGDMSTTRE--- 683
               ... ..               : :.                  . : ..   *   :
```

```
Ta BOR1.2      PK 712
Ta BOR1.3      PK 712
Ta BOR1.1      PK 712
Os BOR1        PK 711
At BOR1        N- 704
At BOR4        --
```

Figure 2-1. Sequence alignment and phylogenetic analysis of BOR-like proteins in wheat, *A. thaliana* and rice.

(A) Amino acid sequence similarity between BOR1-like proteins in *A. thaliana*, rice and wheat; At BOR1, Os BOR1, Ta BOR1.1, Ta BOR1.2 and Ta BOR1.3, and At BOR4 protein in *A. thaliana*. The black arrows indicate the positions of amino acid residue which are different among Ta BOR1.2 and others.

(B)

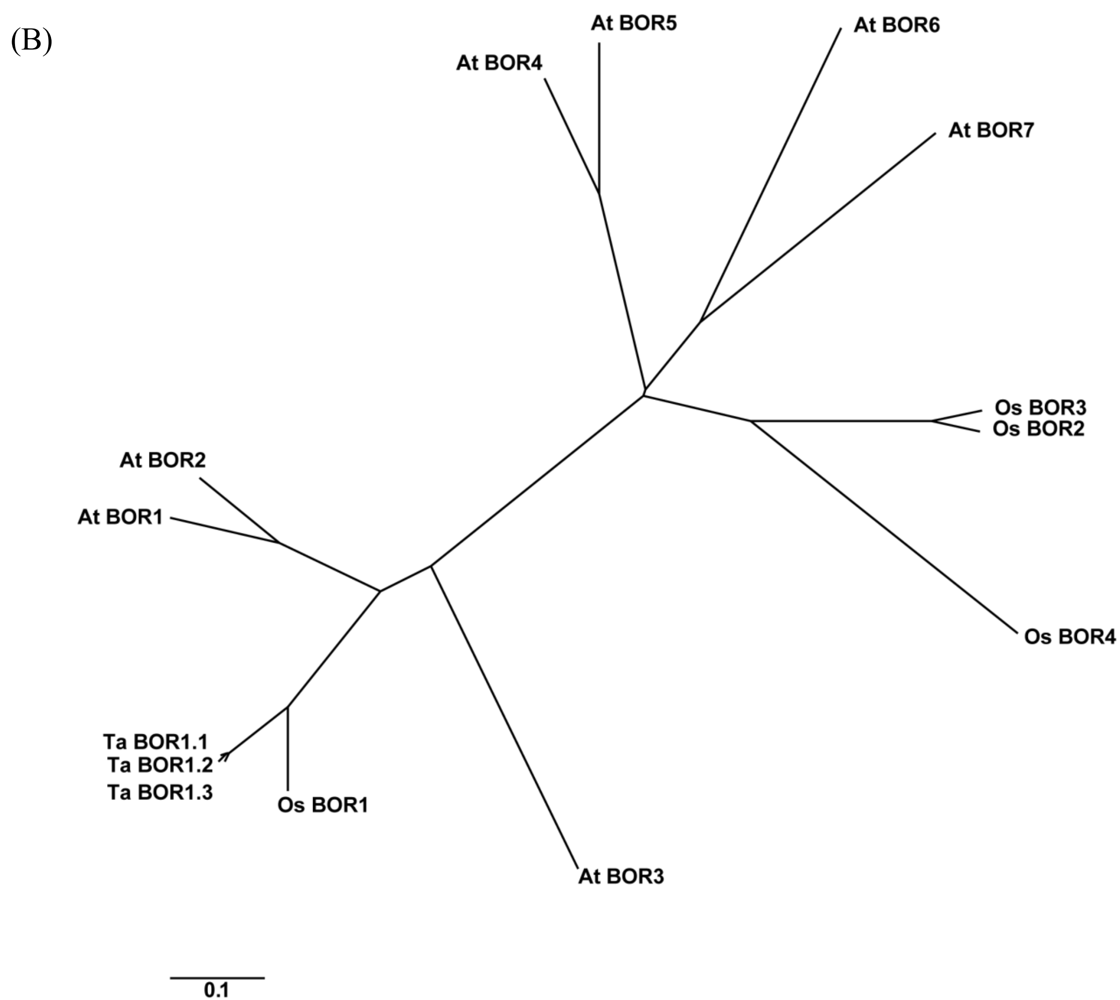


Figure 2-1. Sequence alignment and phylogenetic analysis of BOR-like proteins in wheat, *A. thaliana* and rice.

(B) Phylogenetic tree of wheat, *A. thaliana* and rice BOR-like proteins. Phylogenetic analysis was performed with clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Accession number: At2g47160 for At *BOR1*; At3g62270 for At *BOR2*; At3g06450 for At *BOR3*; At1g15460 for At *BOR4*; At1g74810 for At *BOR5*; At5g25430 for At *BOR6*; At4g32510 for At *BOR7*; Os12g37840 for Os *BOR1*; Os01g08040 for Os *BOR2*; Os01g08020 for Os *BOR3* and Os05g08430 for OS *BOR4*.

2.3.2 B transport activity of Ta BOR1s (by Dr. T. Fujibe)

To examine the B transport activity of Ta BOR1s, the corresponding cDNAs were stably expressed in BY-2 cells under the control of pUbi. To obtain stably transformed BY-2 cells, cells were co-cultured with *Agrobacterium* carrying vectors for transformation followed by selection by Kanamycin/Hygromycin on the solid media. After the selection, transformants on a dish were collected and brought back to the liquid culture before B uptake studies. The transformed BY-2 cells in these experiments are mixtures of a number of independently transformed cells.

Cells were incubated for 60 min in the medium containing 1 mM boric acid and the B concentrations in the cells were determined by ICP-MS. After the one hour incubation, the B concentration of BY-2 cells transformed with the empty vector was 13.9 nmol/mg dry weight, whereas the B concentrations in cells expressing Ta BOR1.1, Ta BOR1.2 and Ta BOR1.3 were 5.8, 7.2 and 8.6 nmol/mg dry weight, respectively. The B concentrations in BY-2 cells were reduced by 57, 50 and 43% of the vector control by expression of Ta BOR1.1, Ta BOR1.2 and Ta BOR1.3, respectively (Figure 2-2), suggesting that all Ta BOR1s are functional efflux-type B transporters similar to At BOR1 and Os BOR1 (Takano et al., 2002; Nakagawa et al., 2007).

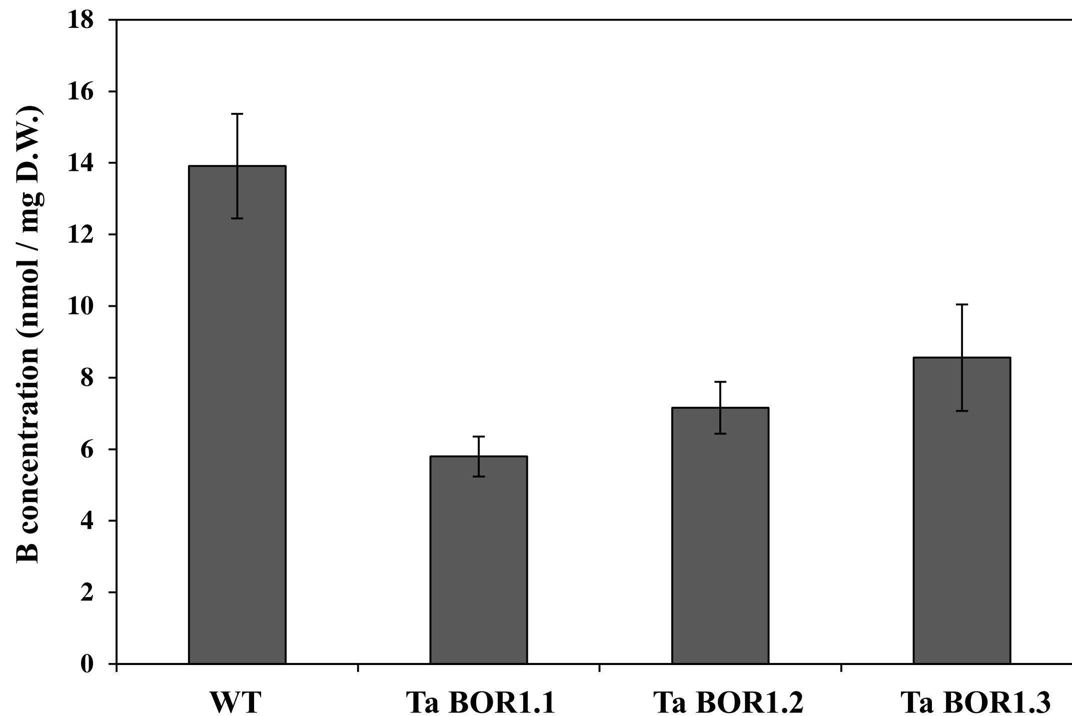


Figure 2-2. B transport activity of Ta BOR1s in BY-2 cells.

B concentrations in BY-2 cells expressing Ta BOR1.1, Ta BOR1.2 and Ta BOR1.3. The B concentrations of vector control (WT) or BY-2 cells carrying pUbi::Ta BOR1.1:GFP (Ta BOR1.1), pUbi::Ta BOR1.2:GFP (Ta BOR1.2) or pUbi::Ta BOR1.3:GFP (Ta BOR1.3) are shown. Cells were exposed for 60 min to medium containing 1 mM boric acid. DW, dry weight.

2.3.3 Subcellular localization of Ta BOR1s in *A. thaliana* leaf cell. (by Mr. S. Wang)

To investigate the subcellular localization of Ta BOR1s, open reading frames (ORFs) of Ta BOR1.1 to 1.3 were fused to the 5' end of ORF of green fluorescent protein (GFP) and placed under the control of an ubiquitin1 promoter (pUbi::Ta BOR1s:GFP). NIP5;1 fused to the 3' end of ORF of mCitrine under the control of an ubiquitin10 promoter (pUBQ10::mCitrine-NIP5;1, Alassimone et al., 2010) was used as a plasma membrane marker. The constructs were introduced into *A. thaliana* leaf cells by *Agrobacterium*. The leaves were incubated with FM4-64 to visualize the plasma membrane before observation by a confocal laser scanning microscope. GFP fluorescence were observed in cell periphery similar to FM4-64 of cells expressing Ta BOR1.1-GFP (Figure 2-3A), BOR1.2-GFP (Figure 2-3B), BOR1.3-GFP (Figure 2-3C) and mCitrine-NIP5;1 (Figure 2-3E). In leaves co-cultivated with *Agrobacterium* with or without constructs (negative control; Figure 2-3D), the stomata in the guard cells showed green autofluorescence. Although some cells of the negative control showed green autofluorescence in the cell periphery, the signals in all Ta BOR1s-GFP expressing cells were stronger than that of autofluorescence, suggesting that Ta BOR1.1 to 1.3 are localized to the plasma membrane.

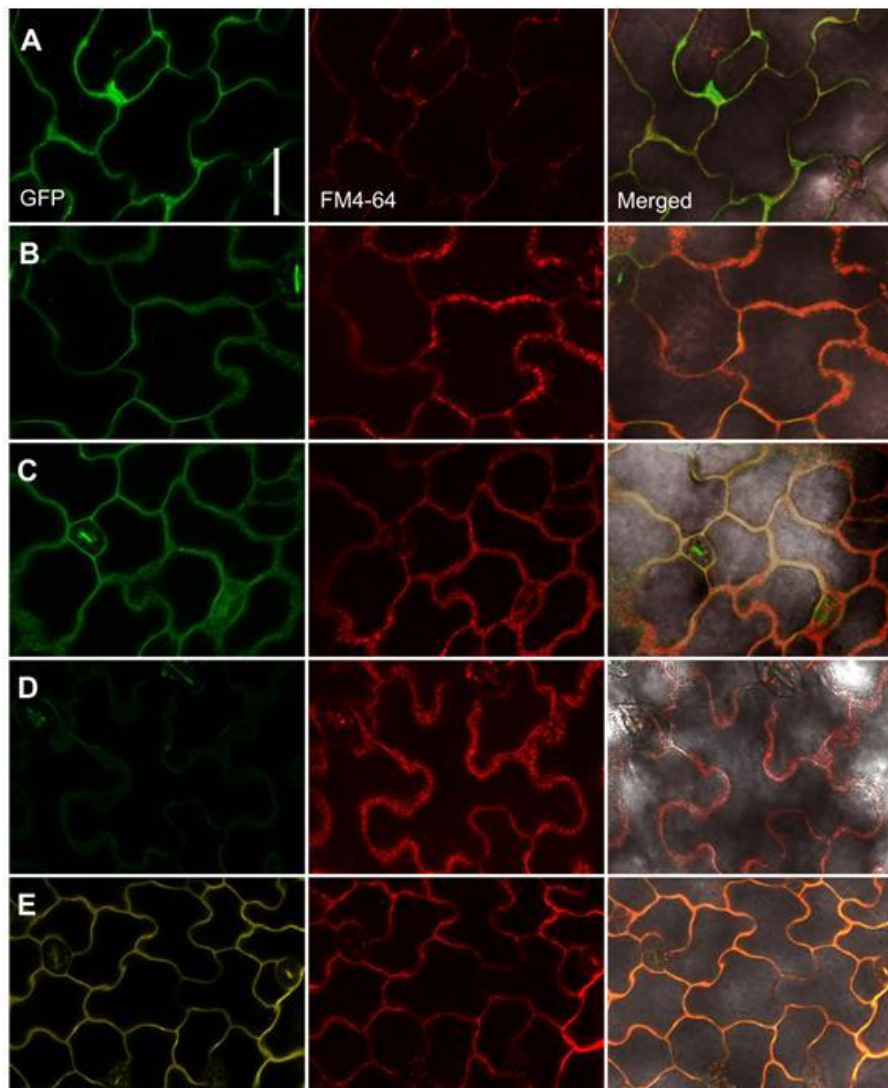


Figure 2-3. Subcellular localization of Ta BOR1s:GFP in *A. thaliana* leaf cells.

Confocal microscopy images of the *A. thaliana* leaf cell expressing Ta BOR1s:GFP are shown. The plasma membrane was stained with FM4-64. A, *A. thaliana* leaf cell expressing pUbi::Ta BOR1.1:GFP. B, *A. thaliana* leaf cell expressing pUbi::Ta BOR1.2:GFP. C, *A. thaliana* leaf cell expressing pUbi::Ta BOR1.3:GFP. D, Negative control (*A. thaliana* leaf cell). E, *A. thaliana* leaf cell expressing pUBQ10::mCitrine-NIP5;1. Left, GFP fluorescence; middle FM4-64 fluorescence; right, merged image. Bars = 10 μm.

2.3.4 Patterns of Ta *BOR1*s transcript accumulation: tissue specificity and response to B conditions

The patterns of transcript accumulation of Ta *BOR1.1*, Ta *BOR1.2* and Ta *BOR1.3* were examined by real-time PCR. Roots and shoots were harvested from wheat grown hydroponically under low B (18 nM B), sufficient B (18 µM B) or excess B (1 mM B) conditions for 18 days. Total RNA was extracted from roots and shoots of the samples and the copy number of the transcript present in the total RNA samples was determined (Figure 2-4). Ta *BOR1* genes were expressed in both roots and shoots with different abundances and patterns in response to B (Figure 2-4). In roots, abundance of transcripts for Ta *BOR1.1*, Ta *BOR1.2* and Ta *BOR1.3* were more or less equivalent, while in shoots, transcript for Ta *BOR1.2* is the most abundant followed by those of Ta *BOR1.1* and Ta *BOR1.3*. Ta *BOR1.3* transcript accumulation is much less than the others in shoots. The three genes responded differently to B conditions. Accumulation of Ta *BOR1.1* mRNA was induced in both roots and shoots by B deficiency. The expression levels of Ta *BOR1.1* was 1.6 and 4.7 times higher in roots and was 2.4 and 3.3 times higher in shoots under low B condition than under sufficient or excess B conditions, respectively. In contrast, Ta *BOR1.2* gene was expressed at high levels in excess B conditions in roots and shoots. Ta *BOR1.2* transcript level in roots in the excess B condition was 1.9 times higher than those under low and sufficient B conditions. Ta *BOR1.2* transcript level in shoots under excess B condition was 1.9 times higher than that under the low B condition. The expression of Ta *BOR1.3* gene was induced mainly in roots. The accumulation of Ta *BOR1.3* transcript in low or sufficient B conditions was 2.3 and 2.7 times higher in roots and 1.6 and 2.8 times higher in shoot

than that in excess B conditions, respectively. These results indicated that the accumulation of Ta *BOR1*s are regulate at transcriptional level. Ta *BOR1.1* and Ta *BOR1.3* responded to B deficiency, while Ta *BOR1.2* responded to B toxicity in both roots and shoots.

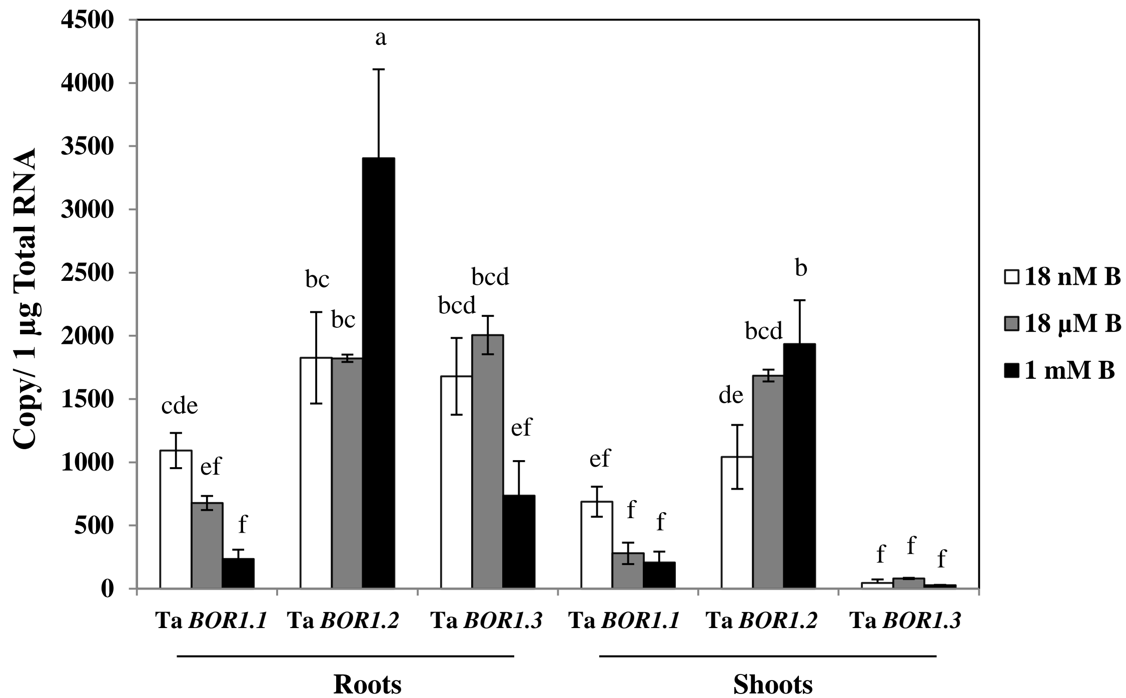


Figure 2-4. Accumulation of Ta *BOR1* transcripts in roots and shoots in response to B conditions.

Plants were grown in low B (18 nM B; white bars), sufficient B (18 µM B; grey bars) or excess B (1 mM B; black bars) hydroponic solutions for 18 d. Average copy number of Ta *BOR1.1* mRNA, Ta *BOR1.2* mRNA or Ta *BOR1.3* mRNA is shown for roots and shoots. Means of three biological replicates±SE for each treatment are shown (n=3). The different letters above each bar represent statistically significant differences (analysis of variance, $p < 0.01$, post-hoc Tukey's test).

2.4 Discussion

2.4.1 Ta BOR1s are orthologs of Os BOR1 and At BOR1

At BOR1 and Os BOR1 are efflux-type B transporters in *A. thaliana* and rice, respectively (Takano et al., 2002; Nakagawa et al., 2007). In this study, Ta BOR1s were identified as wheat efflux transporters of B by Dr. Fujibe. The phylogenetic analysis showed that the three *BOR1*-like genes (Ta *BOR1.1*, Ta *BOR1.2*, and Ta *BOR1.3*) in the wheat genome are the most similar to Os *BOR1* (Figure 2-1B). Ta BOR1s show 91 and 79-80% amino acid identity with Os BOR1 and At BOR1, respectively. The close phylogenetic relationships of the Ta BOR1 genes and functional similarities of the gene products indicate that Ta BOR1s are orthologs to Os BOR1 and At BOR1.

In the present study, Dr. Fujibe used tobacco BY-2 cells for the functional study of the transporters. Ta *BOR1s* are capable of reducing the B concentrations in cells when expressed in tobacco BY-2 cells (Figure 2-2). Furthermore, all Ta BOR1s-GFP expressed in *A. thaliana* leaf cells showed localization to cell periphery (Figure 2-3). Considering that the Ta BOR1s are predicted to have multiple transmembrane domains, it is most probable that these transporters are localized in the plasma membrane, similar to the case of At BOR1 and Os BOR1 (Takano et al., 2002; Nakagawa et al., 2007). Taken together, these results suggest that Ta BOR1s are efflux-type B transporters in plant cells.

2.4.2 The expression of three Ta *BOR1* in roots and shoots are differentially affected by B conditions

In the present study, I demonstrated that three Ta *BOR1* genes are expressed with a different tissue-specificity and showed different responses to B conditions (Figure 2-4). This is a clear contrast to the previously reported cases in *A. thaliana* and rice. The accumulation of At *BOR1* and Os *BOR1* transcripts in leaves and roots were not affected by B limitation (Takano et al., 2010, Nakagawa et al., 2007). Apparently, the regulation of Ta *BOR1*s is different from the cases of At *BOR1* and Os *BOR1*, suggesting the different roles and regulation of these genes.

Recently, six *BOR1*-like genes were identified in *Brassica napus* named as Bn *BOR1;1a,* Bn *BOR1;1c*, Bn *BOR1;2a,* Bn *BOR1;2c*, Bn *BOR1;3a* and Bn *BOR1;3c* (Sun et al., 2012). Semi-quantitative real time PCR analysis of these Bn *BOR1s* showed that Bn *BOR1;1a*, Bn *BOR1;1c,* Bn *BOR1;2a*, and Bn *BOR1;2c* were mainly expressed in root, stem, and flower, while Bn *BOR1;3a* and Bn *BOR1;3c* were expressed in all tested tissues, including root, stem, leaf, flower, bud, and silique. The accumulation of Bn *BOR1;1c* and Bn *BOR1:2a* mRNA increased under low B conditions compared to under high B conditions, while the accumulation of mRNA in the rest of Bn *BOR1s* were not changed and constitutively expressed both under low and high B conditions. In the case of Ta *BOR1s*, the accumulations of Ta *BOR1.1* and Ta *BOR1.3* mRNA were up-regulated in response to B limitation (Figure 2-4). In contrast, the accumulation of Ta *BOR1.2* mRNA increased under excess B condition compared to low and normal B condition in roots. Considering that *Brassica napus* is amphidiploid, it is likely that expression patterns of *BOR1* genes in a plant species with complex genomes differ

among the members of the *BOR1* genes. It is likely that these genes are diversified and have different physiological roles.

2.4.3 Possible functional differences among the Ta BOR1s

Although the accumulation of Os *BOR1* transcripts in whole roots is not affected by B limitation, the histochemical observation of *p*Os *BOR1:GUS* transgenic plants demonstrated that the expression of Os *BOR1* in endodermal and exodermal cells responded to B limitation (Nakagawa et al., 2007). Ta BOR1 proteins are very similar to Os BOR1. It is possible that Ta BOR1s have differential tissue-specific expression, although the tissue-specific expression of Ta BOR1s is required analyzed further. Interestingly, the accumulation of Ta *BOR1.2* transcript was at high levels under excess B condition in both roots and shoots, suggesting that Ta BOR1.2 may have a function to exclude B from tissues for resistance to B toxicity as is the case of At BOR4 (Miwa et al., 2007). At BOR4 is a paralog of At BOR1, and has different characteristics from At BOR1 in terms of polar localization and protein degradation in response to B. At BOR1 is degraded via the endocytic pathway in response to B, while At BOR4 is not (Miwa et al., 2007). Overexpression of At BOR4 showed B-toxicity tolerance and reduced the B concentration in roots and shoots under excess B conditions compared to wild type (Miwa et al., 2007). To investigate the possibility that Ta BOR1.2 has a difference function from Ta BOR1.1 and Ta BOR1.3, I compared the amino acid sequences among Ta BOR1s, Os BOR1, At BOR1, and At BOR4 (Figure 2-1A). I found that there are 3 positions of amino acid residue that are different among Ta BOR1.2 and others. It is possible that these positions of Ta BOR1.2 might be importance for B toxicity tolerance.

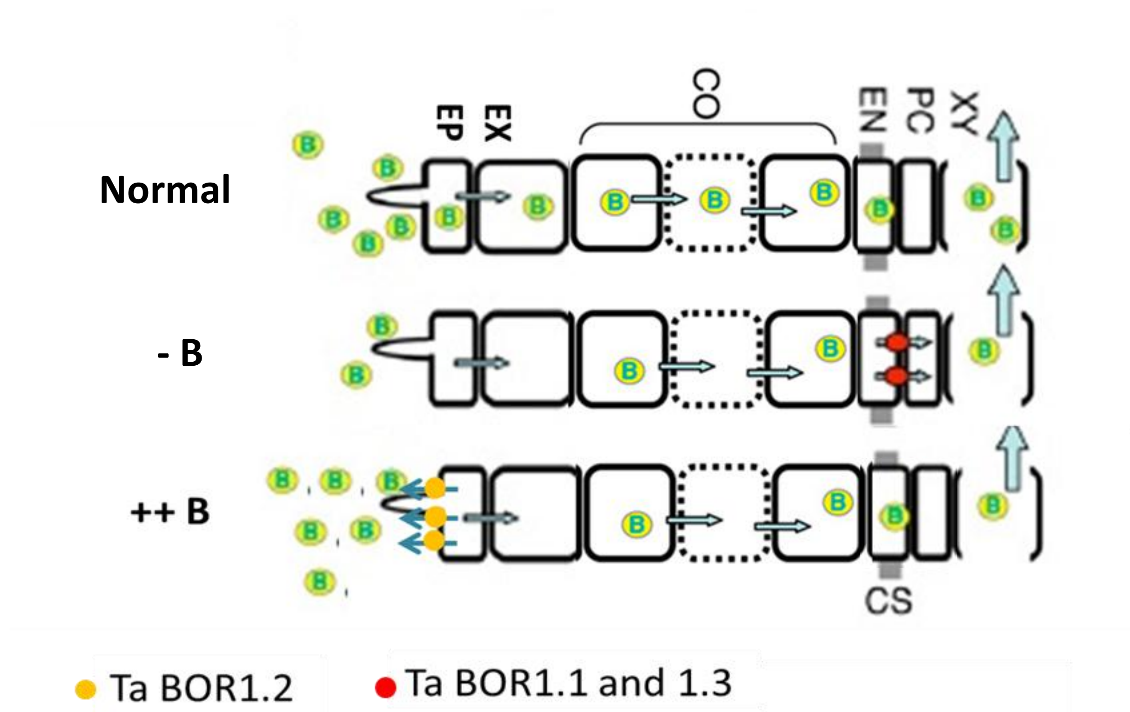


Figure 2-5. Model of the possible roles of Ta BOR1s in roots under normal, low (-B) and excess (++B) B conditions. The diagram shows the cell layers in roots, with the epidermis at the left side and xylem vessels to the right. Grey boxes indicate the positions of the Casparian strips. In wheat, Ta BOR1.1 and Ta BOR1.3 may play the same role as At BOR1 that involved in xylem loading of B in roots under -B condition. In contrast, Ta BOR1.2 may have a function to exclude B from roots to soil under ++B as is the case of At BOR4. CS, Casparian strip; EN, endodermis; CO, cortex; SC, sclerenchyma cells; EX, exodermis; EP, epidermis; PC, pericycle; XY, xylem. (Modified from Nakagawa et al., 2007)

In summary, the wheat genome contains three *BOR1*-like genes, named Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* (Figure 2-1). The Ta BOR1 is most similar to Os BOR1 (91% amino acid identity). The Ta BOR1s are efflux-type B transporters (Figure 2-2) that localize to the plasma membrane (Figure 2-3). The accumulation of Ta *BOR1*s are regulated at transcriptional level. Ta *BOR1.1* and Ta *BOR1.3* transcripts are up-regulated under low B conditions, while Ta *BOR1.2* transcript is up-regulated under excess B condition (Figure 2-4). Based on the transcript accumulation in wheat, Ta BOR1.1 and Ta BOR1.3 may play a role in xylem loading of B under low B condition. Ta BOR1.2 may exclude B from roots to soil under excess B condition (Figure 2-5). These expression patterns and possible functional differences represent an interesting example for the diversification of very similar genes in plant species with complex genomes.

CHAPTER 3

Comparison of *BOR1*-Like Gene Expression in Two Genotypes with Different B Efficiencies in Wheat (*Triticum aestivum* L.), Maize (*Zea mays* L.) and Rice (*Oryza sativa* L.) in Thailand

3.1 Introduction

Genotypic variation in the response to low B supply has been reported in wheat maize, rice, and barley. A large variation among genotypes has been reported in term of the response to low B condition. These genotypes were classified into different B efficiency tolerant or susceptible to B deficiency (Rerkasem and jamjod, 1997a; Jamjod and Rerkasem, 1999; Wongmo et al., 2004; Lordkaew eat al., 2011; Lordkaew et al., 2013). To advance the understanding of molecular basis of B efficiency, *BOR1*-like gene expression in wheat, maize and rice was determined between two genotypes of each crop.

Here, I presented a comparative investigation of the differential transcript levels of *BOR1*-like genes expressed in roots, leaves, and reproductive organs using quantitative real-time PCR. Two genotypes with different B efficiencies in wheat (*Triticum aestivum*), maize (*Zea mays*) and rice (*Oryza sativa*) were grown under B deficiency in Thailand.

3.2 Materials and Methods

3.2.1 Plant materials and growth condition

This part was carried out at Chiang Mai University in Thailand (latitude 18º47'N, longitude 98º59'E, altitude 330 m above mean sea level) in 2011-2012. Two varieties of each species, Fang60 (B-efficient variety) and Bonza (B-inefficient variety) for wheat; Sweet corn (B-efficient variety) and NS72 (B-inefficient variety) for maize; Suphanburi1 (SPR1; B-efficient variety) and Chainart1 (CNT1; B-inefficient variety) for rice, were used in this experiment. The experiment was based on a randomized complete block (RCB) model with two levels of B treatments replicated three times each, with one separate set of pots for each of the two harvests (vegetative and reproductive stage). Set of two plants was sown in the pots (0.30 m diameter and 0.30 m deep) filled with washed quartz river sand. The pots were watered twice daily with 1 L of a complete nutrient solution with two levels of B (0 or 10 µM B for wheat and rice; 0 or 20 µM B for maize). The nutrient solution was adapted from Broughton and Dilworth (1997) and Mozafar (1989) and consisted of: 15 mM KNO_3, 1 mM $CaCl_2$, 2 mM $Mg(SO_4)_2$, 1 mM KH_2PO_4, 100 µM Fe-EDTA, 250 µM K_2SO_4, 9 µM $MnSO_4$, 0.76 µM $ZnSO_4$, 0.31 µM $CuSO_4$, 0.1 µM $CoSO_4$, and 0.1 µM Na_2MoO_4.

Plants were harvested during vegetative (1 month old roots and leaves) and reproductive growth stages (roots, leaves and ear for wheat; roots, leaves, baby corn and ear for maize; and roots, leaves, and panicle for rice). Then, the collected samples were stored at -80 ºC until RNA extraction.

3.2.2 RNA extraction

Total RNA was extracted from plant tissues that were harvested as described above. The method was followed from Laksana (2011). Each tissue was grounded in liquid nitrogen to a fine powder, then transferred into a microcentrifuge tube and added 600 µl of RNA extraction buffer (2% CTAB, 2% PVP, 2M $NaCl_2$, 25mM EDTA, 100 mM Tris-HCl pH 8.0 and 2% α-mercaptoethanol (v/v)), and vortexed vigorously. The mixture was incubated for 10 min at room temperature and then 600 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added followed by mixing by inverting tube. The mixture was centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and added 1 volume of chloroform: isoamyl alcohol (24:1), and mixed by inverting tubes. The mixture was centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was transferred to a new microcentrifuge tube and 400 µl of isopropanol and 100 µl of $NaCl_2$ was added to the supernatant, mixed by inverting tube and incubated for 10 min at -20°C. After that, the mixture was centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was discarded and washed the pellet with 400 µl of cold 70% ethanol. The mixture was centrifuged at 12,000 xg for 3 min at 4°C. The supernatant was removed, and the pellet was then dried out and resuspended in 20 µl of DEPC-water. The genomic DNA was removed by RNA samples treatment with DNase I and stored at -80ºC.

3.2.3 Quantitative real-time PCR

Total RNA was reverse-transcribed to cDNA using the PrimeScript RT reagent kit with Oligo$(dT)_{16}$ primer. The real-time PCR amplification was performed using a

Thermal Cycler Dice with SYBR Premix Ex Taq II (Takara). The *VIP2*, *HMG* or *Ubi1* genes were used as an internal control for quantitation in wheat, maize, or rice, respectively. The specific primers for *BOR1*-like genes were designed using Primer3 software (http://frodo.wi.mit.edu/primer3). The sequences of the primers were shown in Table 3-1.

Table 3-1. Genes and gene-specific primers used for the real-time PCR experiments

Plant	Gene name	Oligonucleotide primer sequences used for real-time PCR, 5´to 3´	
		Forward	Reverse
Wheat	Ta *BOR1.1*	ATACGCTCTCCGATTCCTTC	AAGCATCGCGATGAGGAGCCCG
	Ta *BOR1.2*	AAAGCGTAATCTGGGTCTCT	GCTGCATGAAGAGCATTGCG
	Ta *BOR1.3*	GCGTTTCTCCCGGTCCGGTC	TGATGACCGTGGGCTCGGCC
	Ta *VIP2*	GGGCGCATCTGCCCCATTCC	GGCCAGATGAACCCGGTGGG
Maize	Zm *BOR1*	TGATGGAGTCCTCAGAGCAG	GTATGGCCAGCAAGAAGAGC
	HMG	GCTACATAGGGAGCCTTGTCCT	TTGGACTAGAAATCTCGTGCTGA
Rice	Os *BOR1*	CACTAGAAGCCGTGGTGAAA	CAGGTAGTTGCATAGCTCAT
	Ubi1	GACAAGGAGGGAATCCCG	GCATAGCATTTGCGGCA

3.3 Results

3.3.1 Transcript levels of Ta *BOR1* genes among two genotypes of wheat related to B deficiency

To determine the expression levels of *BOR1*-like gene between the two genotypes in response to B status, the transcript levels of Ta *BOR1.1*, Ta *BOR1.2* and Ta *BOR1.3* were comparatively analysed between B-efficient (Fang 60) and B-inefficient (Bonza) wheat genotypes in different organs, including roots (vegetative stage), leaves (vegetative stage), roots (reproductive stage), leaves (reproductive stage) and ear under low and sufficient B conditions by using quantitative real-time PCR.

In roots at the vegetative stage, the expression levels of all Ta *BOR1s* showed no statistically difference between those under low B condition and sufficient B condition in both Fang60 and Bonza (Figure3-1).

In leaves at the vegetative stage, the expression levels of all Ta *BOR1s* under B deficiency were lower than those under sufficient B condition in Bonza, while the expression levels of all Ta *BOR1s* in Fang60 under B deficiency were no different as compared with those under sufficient B condition (Figure 3-2).

In roots at the reproductive stage, the expression levels of Ta *BOR1.1* and Ta *BOR1.3* in Fang60 were up-regulated, while the expression levels of Ta *BOR1.2* was down-regulated under low B condition. In case of Bonza, the expression levels of all Ta *BOR1s* were down-regulated in B deficiency (Figure 3-3).

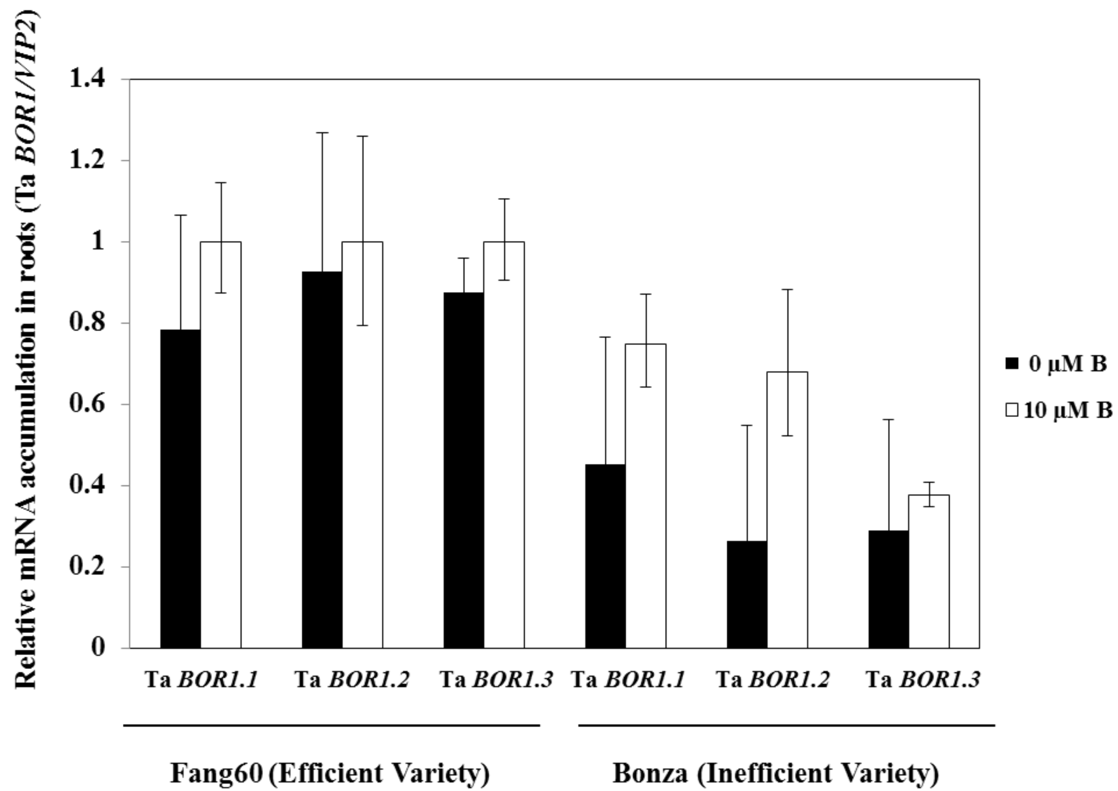


Figure 3-1. Transcript levels of **wheat *BOR1*-like genes**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* **in roots at the vegetative stage; 1 month** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition. The transcript levels of each gene in Fang60 at 10 µM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test.

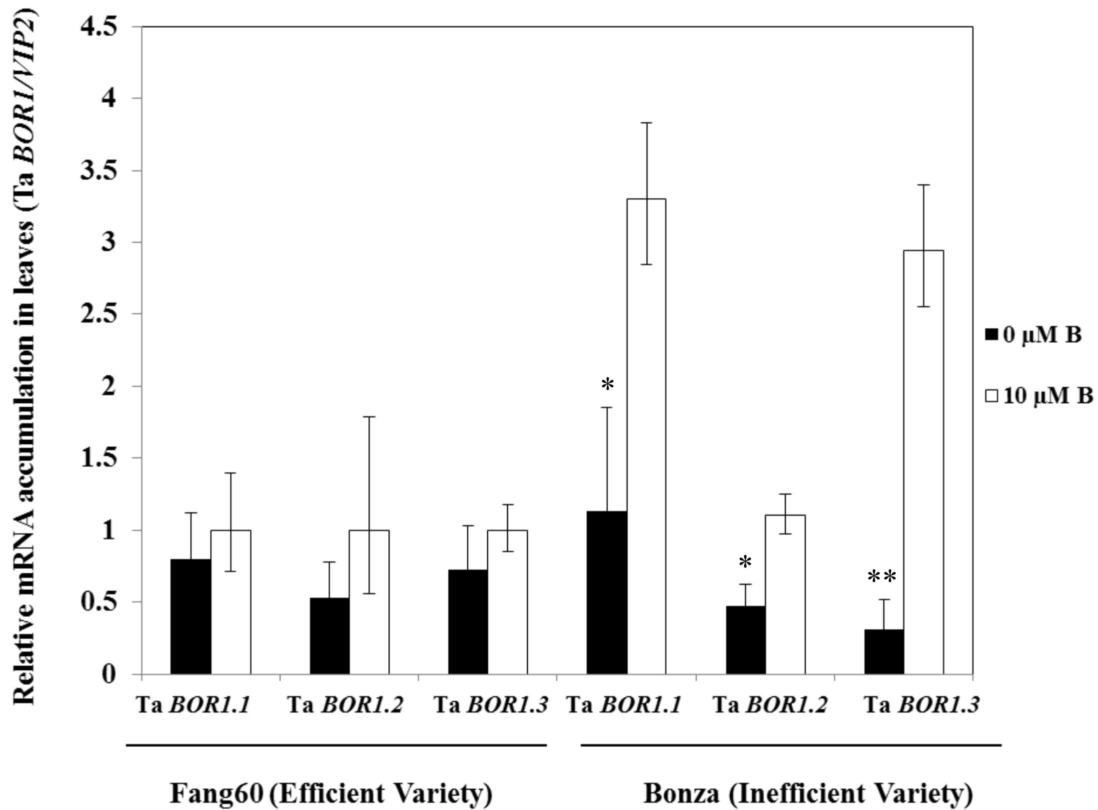


Figure 3-2. Transcript levels of **<u>wheat *BOR1*-like genes</u>**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* **<u>in leaves at the vegetative stage; 1 month</u>** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 μM B) or sufficient (10 μM B) condition. The transcript levels of each gene in Fang60 at 10 μM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * P< 0.05, ** P< 0.01.

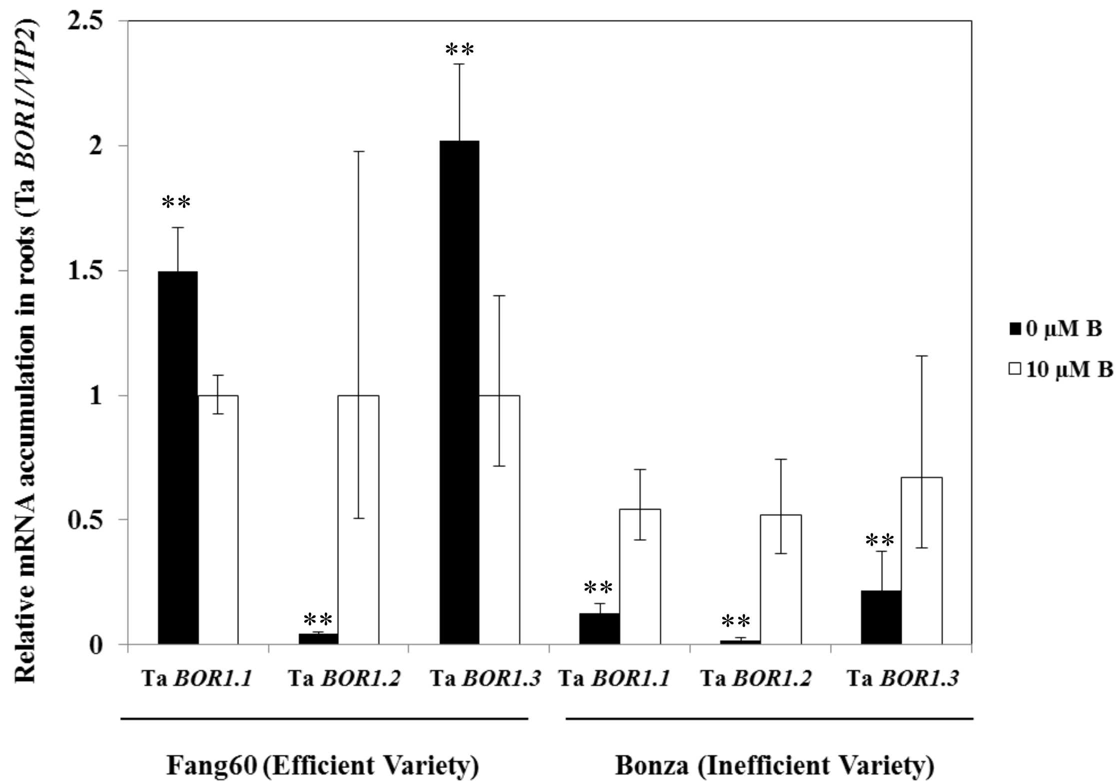


Figure 3-3. Transcript levels of **wheat *BOR1*-like genes**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* **in roots at the reproductive stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition. The transcript levels of each gene in Fang60 at 10 µM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; ** *P*< 0.01.

In leaves at the reproductive stage, the expression levels of Ta *BOR1.3* was up-regulated, while Ta *BOR1.2* expression was down regulated under low B condition in Fang60. Whereas, the expression levels of Ta *BOR1.1* did not change among B conditions in Fang60. The expression levels of all Ta *BOR1*s were not significantly different under low and sufficient B conditions in Bonza (Figure 3-4).

The expression levels in ear of all Ta *BOR1*s were not different between low and sufficient B conditions both in Fang60 and Bonza. (Figure 3-5).

The transcript accumulation patterns of all Ta *BOR1*s were also compared between Fang60 and Bonza under B deficient condition. Ta *BOR1s* genes were expressed at relatively high levels in Fang60 compared to Bonza. Except for the expression levels of Ta *BOR1.1* in roots and leaves at the vegetative stage, the expression levels of Ta *BOR1.2* in leaves at the vegetative stage and ear, the expression levels were not significantly different among two genotypes. In Ta *BOR1.3*, except for ear, in Fang60 was higher than that in Bonza (Figure 3-6). These results suggest that Ta *BOR1s* may be required for wheat growth and development, especially in the reproductive stage under B limitation. This implies that these genes may be involved in the tolerance to B deficiency of wheat.

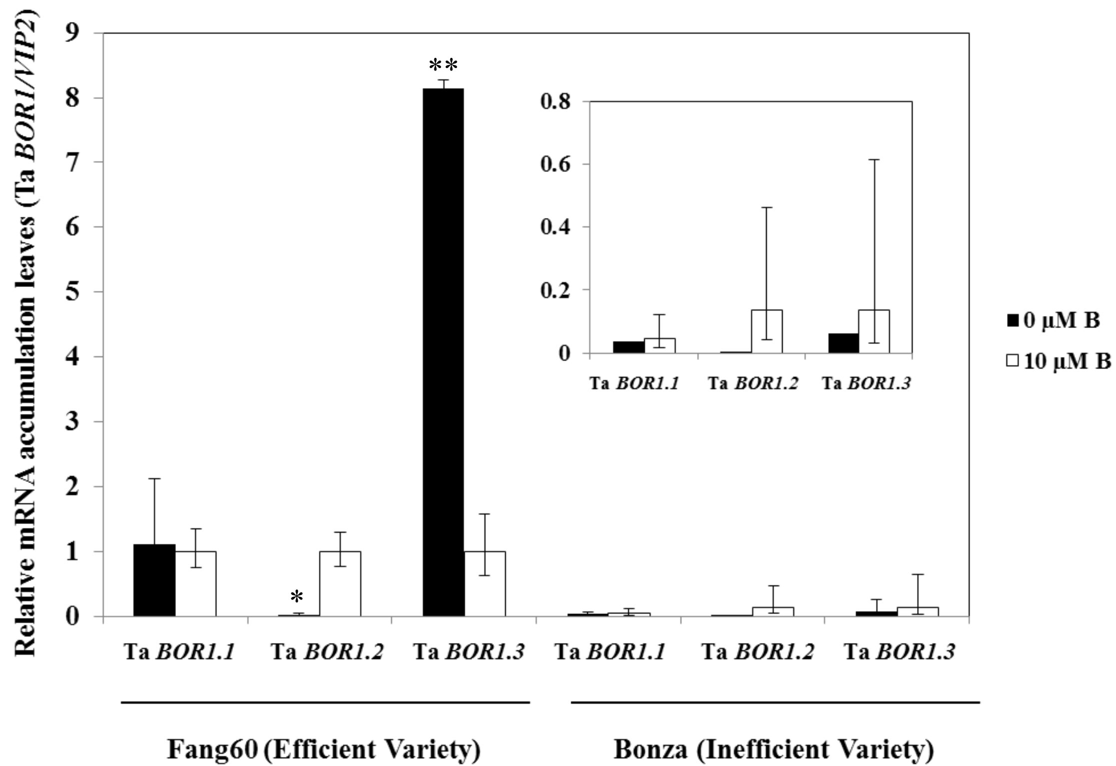


Figure 3-4. Transcript levels of **wheat *BOR1*-like genes**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* **in leaves at the reproductive stage** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition. The transcript levels of each gene in Fang60 at 10 µM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$.

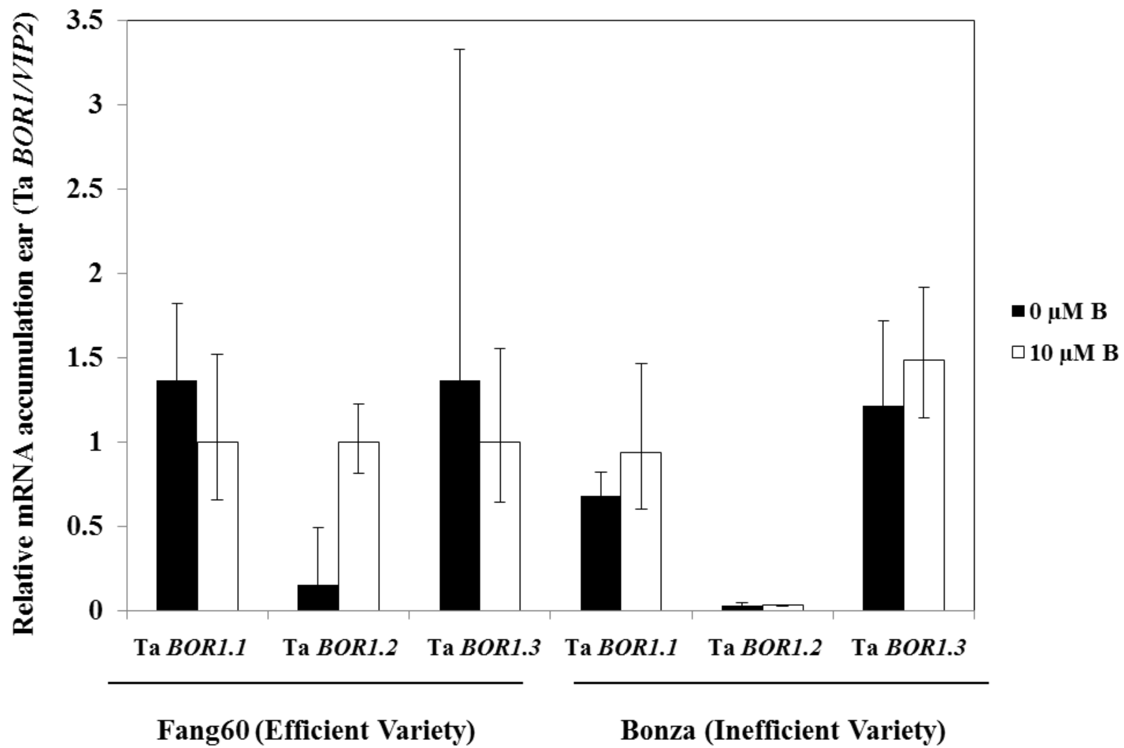


Figure 3-5. Transcript levels of **wheat *BOR1*-like genes**, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* **in ear** of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) condition. The transcript levels of each gene in Fang60 at 10 µM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test.

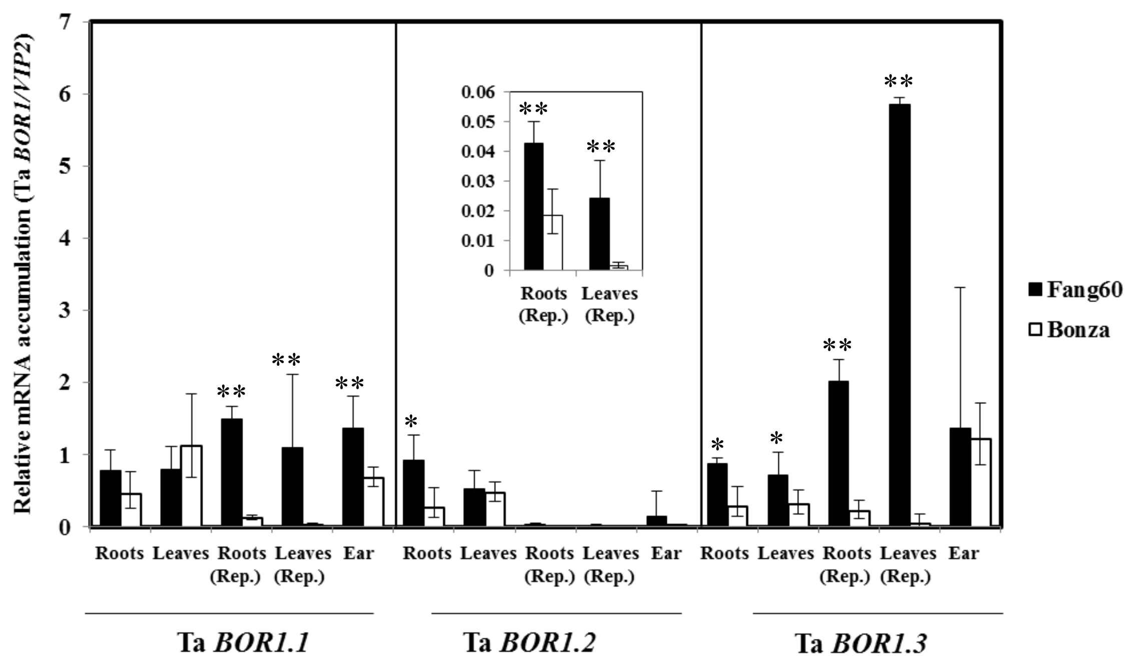


Figure 3-6. <u>Summary of relative expression of wheat *BOR1*-like genes</u>, Ta *BOR1.1,* Ta *BOR1.2* and Ta *BOR1.3* in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage, leaves at the reproductive stage and ear of Fang60 (B-efficient genotype) and Bonza (B-inefficient genotype) under B deficiency were analyzed by real-time PCR. The transcript levels of each gene in Fang60 at 10 μM B were used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$.

3.3.2 Transcript levels of Zm *BOR1* gene among two genotypes of maize related to B deficiency

Similar to wheat *BOR1* genes expression study, Zm *BOR1* genes were compared between B-efficient variety (Sweet corn) and B-inefficient variety (NS72) of maize in different organs including roots (vegetative stage), leaves (vegetative stage), roots (reproductive stage), leaves (reproductive stage), ear and baby corn under low and sufficient B conditions using quantitative real-time PCR. The results demonstrated that Zm *BOR1* of Sweet corn and NS72 mainly expressed in ear (Figure 3-7). In Sweet corn the expression levels of Zm *BOR1* under low B conditions was higher in ear, while in leaves at the vegetative stage, and baby corn, Zm *BOR1* expression was lower than that under sufficient B conditions (Figure 3-7 and 3-8).

The level of transcript accumulation of Zm *BOR1* of NS72 was not significantly different among B conditions, except for baby corn. The expression levels of Zm *BOR1* was up-regulated under low B condition as compared with the sufficient B condition (Figure 3-7 and 3-8).

Zm *BOR1* expression patterns of Sweet corn and NS72 at a various tissues and two developmental stages were comparatively analysed under B deficient conditions (Figure 3-9). The expression levels of Zm *BOR1* in Sweet corn was higher than that in NS72 in ear and roots at both the vegetative and reproductive stage. There was no significantly difference in Zm *BOR1* expression in leaves at both the vegetative and reproductive stages and baby corn between two genotypes (Figure 3-9). These results suggest that the expression of Zm *BOR1* may be correlated with B deficiency tolerance in maize roots.

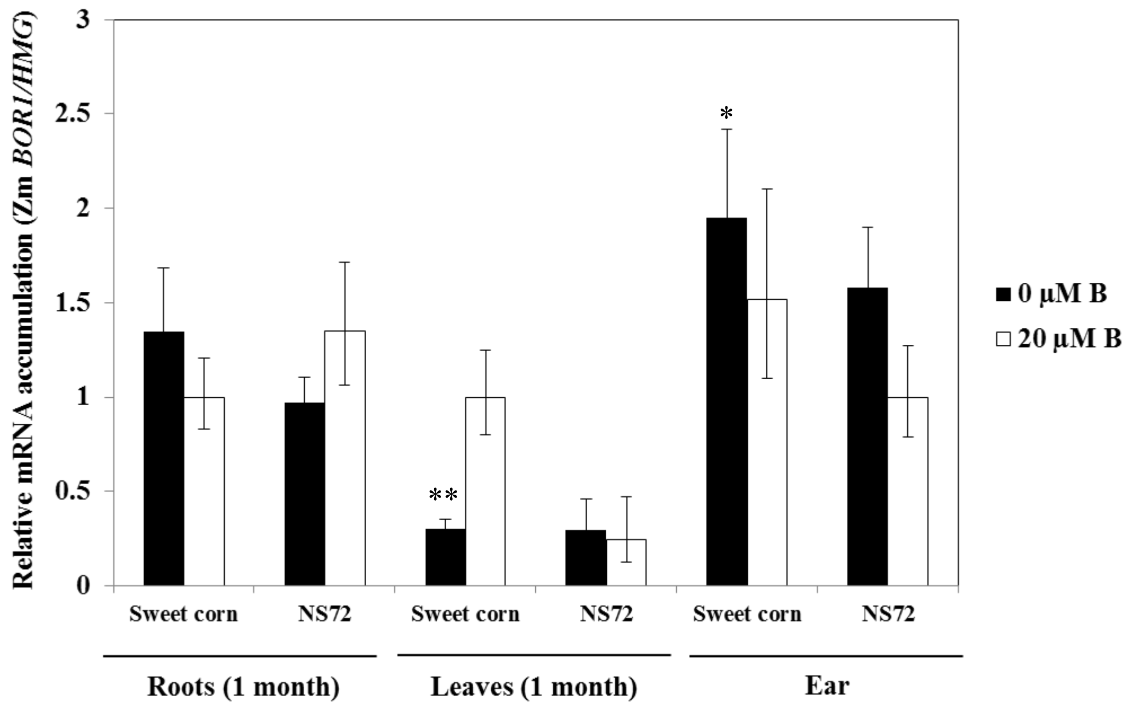


Figure 3-7. Transcript levels of **Zm *BOR1-like* gene in roots and leaves at the vegetative stage, and ear at the reproductive stage** of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficient (0 µM B) or sufficient (20 µM B) condition. The transcript levels of Zm *BOR1* in Sweet corn at 20 µM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$.

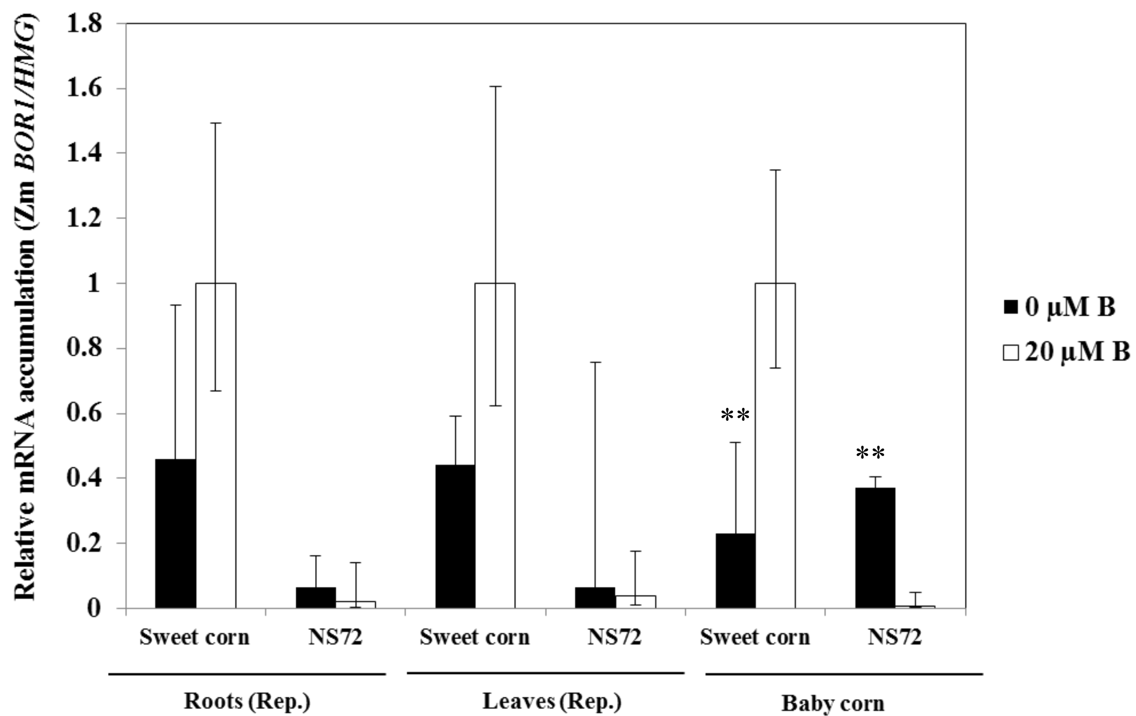


Figure 3-8. Transcript levels of **<u>Zm *BOR1-like* gene in roots, leaves and baby corn at the reproductive stage</u>** of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficient (0 µM B) or sufficient (20 µM B) condition. The transcript levels of Zm *BOR1* in Sweet corn at 20 µM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; ** $P < 0.01$.

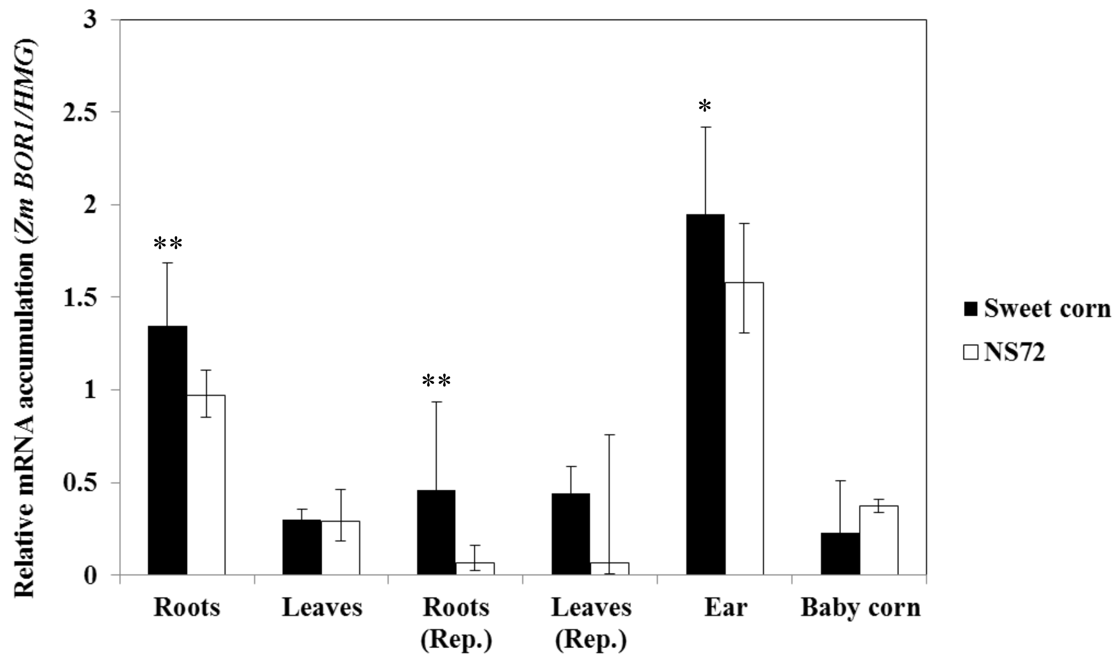


Figure 3-9. Summary of relative expression of Zm *BOR1* in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage, leaves at the reproductive stage, ear and baby corn of Sweet corn (B-efficient genotype) and NS72 (B-inefficient genotype) under B deficiency was analyzed by real-time PCR. The transcript levels of Zm *BOR1* in Sweet corn at 20 μM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$.

3.3.3 Transcript levels of Os *BOR1* gene among two genotypes of rice related to B deficiency

I compared the transcript levels of Os *BOR1* gene between B-efficient variety (Suphanburi1; SPR1) and -inefficient variety (Chainart1; CNT1) of rice.

Similar to the previous results (Nakagawa et al., 2007), the expression levels of Os *BOR1* were not affected by B status in all tested tissues. There were no difference in Os *BOR1* expression among B conditions in both SPR1 and CNT1 (Figure 3-10). In leaves at the reproductive stage and panicle, the expression levels of Os *BOR1* could not be detected under low and sufficient B conditions in CNT1 by using the specific primers for Os *BOR1* as shown in Table 3-1 (Figure 3-10).

The expression patterns of Os *BOR1* gene was compared between SPR1 and CNT1 under B deficient condition (Figure 3-11). Similar to wheat and maize *BOR1*-like gene expression, Os *BOR1* was differentially expressed between B-efficient and B-inefficient varieties. Os *BOR1* gene was expressed at higher level in SPR1 compared to CNT1 (Figure 3-11).

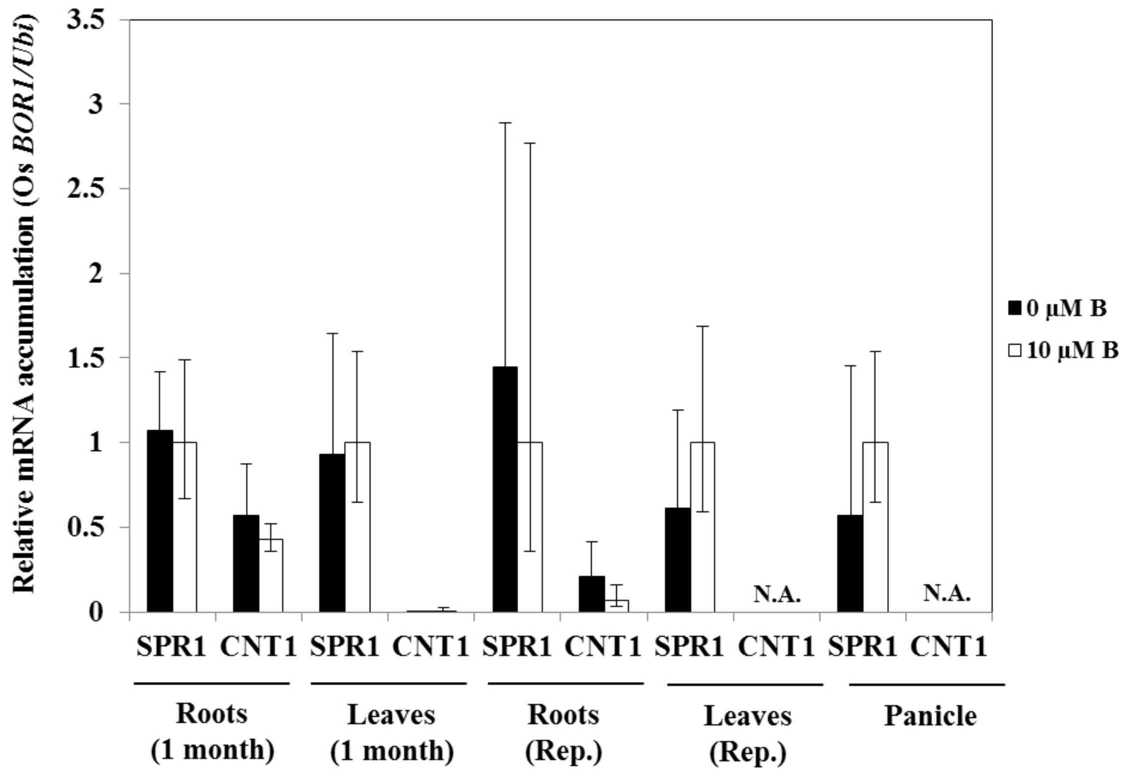


Figure 3-10. Transcript levels of **<u>Os *BOR1* gene in roots and leaves at the vegetative stage, roots and leaves at the reproductive stage, and panicle</u>** of SPR1 (Suphanburi1; B-efficient genotype) and CNT1 (Chainart1; B-inefficient genotype) under B deficient (0 µM B) or sufficient (10 µM B) conditions. The transcript levels of Os *BOR1* in SPR1 at 10 µM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test. N.A., not amplified; Rep., reproductive stage.

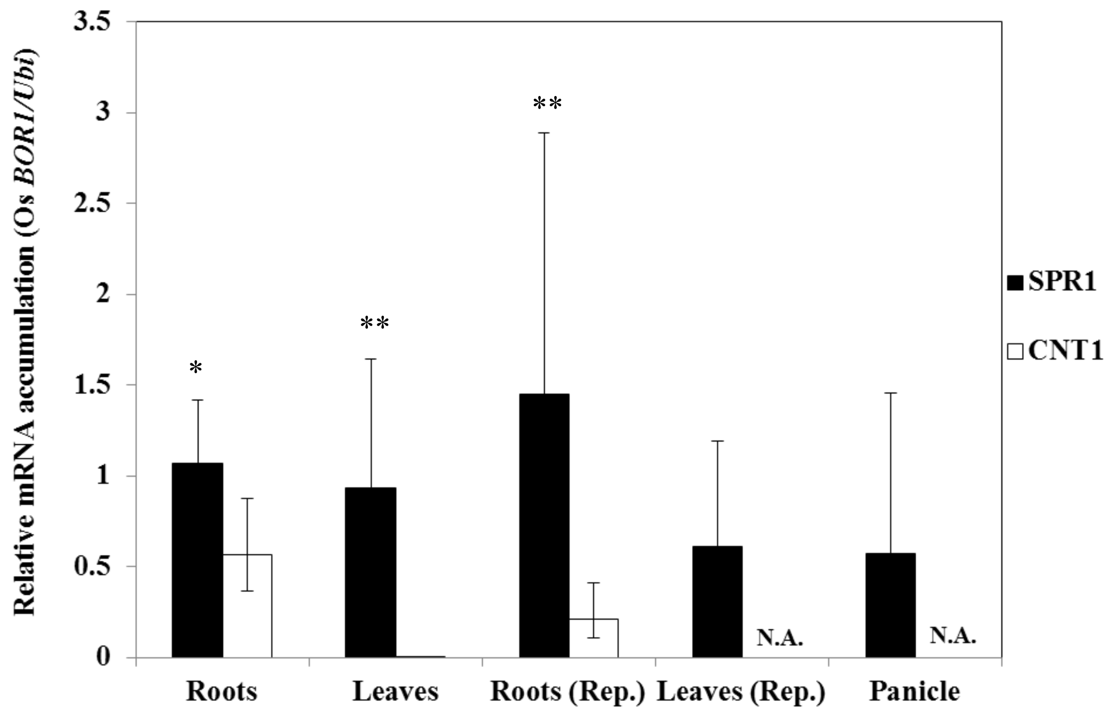


Figure 3-11. Summary of relative expression of Os *BOR1* in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage, leaves at the reproductive stage and panicle of SPR1 (Suphanburi1; B-efficient genotype) and CNT1 (Chainart1; B-inefficient genotype) under B deficiency was analysed by real time PCR. The transcript levels of Os *BOR1* in SPR1 at 10 μM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. N.A., not amplified; Rep., reproductive stage.

3.4 Discussion

In Thailand, the B deficiency in soil is found especially in the north region. B deficiency is observed in wheat, maize, and rice which are grown in the north of Thailand (Rerkasem and Jamjod, 1989). Several researches have been doing to solve the problems. Physiological analysis and genetic variation in B efficiency among plant genotypes have been reported, however, the molecular and genetic mechanisms of B tolerance remain unclear. This study aims to advance the understanding of the molecular basis of B tolerance in wheat, maize, and rice. I compared transcript levels of *BOR1*-like gene, an efflux type B transporters, between B-efficient and -inefficient genotypes in different organs under B deficiency using quantitative real-time PCR.

<u>3.4.1 The expression levels of Ta *BOR1s* genes correlate with the ability of B deficiency tolerance in wheat</u>

The transcript levels of wheat *BOR1*-like gene was comparatively analysed between two genotypes under B limitation. Fang60, which is classified as a B-efficient variety (Jamjod et al., 1992) and Bonza, which is classified as a B-inefficient variety (Rerkasem and Jamjod, 1997a) were used in this study. Three *BOR1*-like genes (named Ta *BOR1.1*, Ta *BOR1.2*, and Ta *BOR1.3*), which is the most similar to Os *BOR1* in amino acid sequence, were identified from wheat genome and these are functional efflux transporters of B like At BOR1 and Os BOR1 (Chapter 2).

Ta *BOR1.1* of Fang60 was expressed at 12, 37 and 2 folds higher as compared those of Bonza in roots at the reproductive stage, leaves at the reproductive stage, and ear, respectively (Figure 3-6). Ta *BOR1.2* of Fang60 was expressed at 4.5, 1.3, 2.0, and

10 folds higher than those of Bonza in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage, and leaves at the reproductive stage, respectively (Figure 3-6). While, Ta *BOR1.3* was expressed at 4.0, 2.3, 10, and 130 folds higher in Fang60 than in Bonza in roots at the vegetative stage, leaves at the vegetative stage, roots at the reproductive stage and leaves at the reproductive stage, respectively (Figure 3-6). The expression levels of three Ta *BOR1s* in Fang60 were high under B deficient condition in most of the tested organs as compared with those in Bonza (Figure 3-6). In wheat, the functional B requirement for reproductive growth is higher than that for vegetative growth (Rerkasem et al., 1997). The critical B concentration required for early vegetative growth in wheat is reported to be about 1 mg B kg^{-1} DW (Asad et al., 2001). In contrast, grain set failure in wheat has been found less than 2-4 mg B kg^{-1} DW in the ear (Rerkasem and Lordkaew, 1992) and 3-7 mg B kg^{-1} DW in the flag leaf at the boot stage (Rerkasem and Loneragan, 1994). Wheat is well known for its difference in their sensitivity to B deficiency for B uptake ability (Cangiani-Furlani et al., 2004 and Nachiangmai et al., 2004). Rerkasem and Jamjod (1997a) observed large variation among wheat genotypes in low B concentrations with quartz river sand experiments. Under low B conditions, the two most sensitive groups of genotypes are completely male sterile and set only a few or no grains, while the tolerant group set grains normally (Cangiani-Furlani et al., 2004). Pollen viability of the B-efficient Fang60 is not affected by withholding B during the critical stage of microsporogenesis, while pollen viability of B-inefficient SW41 is nearly halved. A B-efficient wheat variety may have greater ability to accumulate B from the growing medium than a B-inefficient variety wheat thereby contributing to reproductive development (Nachiangmai et al., 2004). Consistent with this study, Ta *BOR1*s were

differentially expressed between the two genotypes. The expression of Ta *BOR1*s genes are expressed at high levels in tolerant compared to sensitive genotype. It is possible that expression of Ta *BOR1* genes was correlated with the ability of tolerance genotypes to B deficiency in wheat.

Moreover, the results of this study support the previous study. Nachiangmai et al. (2004) determined whether differences in B transport and retranslocation ability among cultivars can explain the differences in B efficiency between B-efficient (Fang60) and B-inefficient (SW4) wheat cultivars. They reported that the ^{10}B accumulation in ears of Fang60 is greater than SW41 with limited external B supply, suggesting that B efficiency was associated with xylem transport of B (Nachiangmai et al., 2004). Furthermore, a study with ^{10}B revealed that Fang60 was able to retranslocate B from older to younger tissues after B supply to the roots was withdrawn, whereas Bonza was not able to (Konsaeng, 2007). The comparative study of Fang60 and SW41 showed that a major mechanism in B efficiency in wheat was associated with the transport of B from roots to the developing ear. *BOR1*, an efflux B transporter, is involved in not only B transport into the xylem but also in B distribution within shoots (Miwa and Fujiwara, 2010). It is possible that the high expression of Ta *BOR1*s enable effective transport from the root into reproductive organs under low B condition.

Most of the Ta *BOR1*s represent the difference in transcript levels between efficient and inefficient varieties under B deficiency. The expression of Ta *BOR1.1,* Ta *BOR1.2,* and Ta *BOR1.3* of Fang60 is higher than that of Bonza in most tested tissues. This study showed that the expression of Ta *BOR1*s genes correlates with the B deficiency tolerance. These genes may be involved in the tolerance to B deficiency of

wheat. Especially, Ta BOR1.1 transcripts in root and leaf at the reproductive stage were higher at both conditions, suggesting that roots and leaves at the reproductive stage are appropriate tissue for B deficiency tolerance selection.

3.4.2 The transcript levels of Zm *BOR1* correlate with the difference in B deficiency in maize

Similar to wheat experiment, this study aimed to evaluate the differences in gene expression patterns between B-efficient and B-inefficient genotypes of maize under B deficiency. The levels of Zm *BOR1* gene transcript were compared in difference organs. Zm *BOR1* is 93% identical in amino acid sequence to Os *BOR1* (Appendix1). In this study, Sweet corn and NS72 were used as a B-efficient and B-inefficient variety, respectively.

Along with most cereals, maize has been generally considered to have a relatively low B requirement compared with other crops (Marten and Westermann, 1991). A wide range of genotypic variation in the B deficiency response has also been reported in maize. Lordkaew et al. (2007) observed the response of maize to B during vegetative and reproductive growth and evaluated genotypic variation in B deficiency response. Seven maize genotypes were evaluated for their response to B. The most tolerant genotype to B deficiency was Sweet corn, which had about the same grain number in B-deficient and -sufficient condition. In contrast, NS1, NS72 and CM are sensitive to B deficiency, which set almost no grain under low B condition.

The Zm *BOR1* mRNA levels were highly expressed in roots at the vegetative stage and higher in Sweet corn than that in NS72, while the expression levels of Zm

BOR1 in leaves was not different between two genotypes (Figure 3-9). These results are consistent with the detailed physiological analysis of two maize genotypes. At seedling stage, the two maize genotypes grew differently in nutrient solution from which B has been removed (Lordkaew, 2007). The concentrations of B in the roots and shoots of Sweet corn were 2-3 folds higher than those of NS72 (Lordkaew et al., 2007). It is likely that the transcript levels of Zm *BOR1* may correlate with the B efficiency in maize genotypes. B-efficient variety would have more effective transport from the root into shoot under low B condition.

The Zm *BOR1* mRNA levels were also highly expressed in ear, while the expression in baby corn is at low level (Figure 3-9). The expression levels of Zm *BOR1* was higher in Sweet corn than in NS72 in roots at the reproductive stage under B deficiency (Figure 3-9). Consistent with the physiological analysis, the B-inefficient genotype appeared more severe symptoms of B deficiency than the B-efficient genotype in roots and leaves at the reproductive stage. Lordkaew et al (2011) demonstrated that the silk was poorly developed and thin under B deficiency in B-inefficient genotypes, whereas silk appeared normal and thick under normal B condition from the part of young ear (baby corn) in both genotypes. It is possible that the higher transcript levels of Zm *BOR1* of Sweet corn in roots at the reproductive stage contributes efficient B uptake and efflux transport from roots to shoots and reproductive organs as compared with NS72. Moreover, roots at the reproductive stage is appropriate tissue for B deficiency tolerance selection.

3.4.3 The expression levels of Os *BOR1* gene correlates with the ability in tolerance to B deficiency of rice

The aim of present study was to investigate how rice genotypes with different B deficient sensitivity, differ with respect to the expression pattern of Os *BOR1* gene, under B deficient condition, which could be related to the varietal difference in tolerance. Genotypic variation in response to low B has been reported in rice. In Pakistan, the yield was depressed range from 9 to 32% in grain and from 2 to 44% in yield due to B deficiency among different rice varieties (Rashid et al., 2002). B deficiency depressed pollen viability and grain set in rice in the same way as in wheat and maize (Rerkasem and Jamjod, 1997b).

SPR1 (Suphanburi1), which is a B-efficient variety, and CNT1 (Chainart1), which is a B-inefficient variety (Lordkaew et al., 2013), were used in this study. Lordkaew et al. (2013) demonstrated that grain weight was strongly depressed by B deficiency, ranging from 28% in SPR1 to 79% in CNT1. B uptake and transporter genes have been identified in rice. As described above, the rice *BOR1* gene, Os *BOR1*, has been identified through phylogenetic analysis (Nakagawa et al., 2007). Os *BOR1* is a B efflux transporter required for normal growth especially under B-limited conditions (Nakagawa et al., 2007).

I compared the expression levels of Os *BOR1* between SPR1 and CNT1 in different organs. The expression levels of Os *BOR1* in all tested organs were not affected by B status. There was no difference in Os *BOR1* expression between low and sufficient B conditions in both SPR1 and CNT1 (Figure 3-10). Similar to the previous results of Nakagawa et al. (2007), the accumulation of Os *BOR1* transcripts in leaves

and roots was not affected by B deprivation. In this study, Os *BOR1* was differentially expressed between SPR1 and CNT1 in all tested organs including roots at both the vegetative and reproductive stage, leaves at both the vegetative and reproductive stage and panicle. The levels of Os *BOR1* transcript accumulation were higher in SPR1 than in CNT1 under low B condition (Figure 3-11). The mechanisms responsible for the different in expression of Os *BOR1* gene remains to be identified. However, this work proved that variation in Os *BOR1* expression between two genotypes is correlated with the B deficiency tolerance in rice.

In summary, to elucidate the molecular basis of B deficiency and tolerance in cereal crops (wheat, maize and rice), the expression analysis of *BOR1*-likes, under B deficiency was performed using quantitative real-time PCR against B-efficient and B-inefficient genotypes. The expression patterns of *BOR1*-like genes are different between two genotypes. *BOR1*-like gene transcripts are accumulated to higher levels in a tolerant cultivar than the sensitive ones in most tested tissues. It is possible that B-efficient variety would have more effective transport of B from the root into reproductive organs. Moreover, *BOR1*-like genes can be useful as an indicator of B deficiency tolerance in wheat maize and rice by selecting appropriate tissues and growth stages.

CHAPTER 4

Analysis of *A. thaliana GLIP1* Gene in Relation to B Deficiency

4.1 Introduction

In recent years, many progresses have been made toward the understanding of molecular mechanisms of B transport in plants (Takano et al., 2002 and 2006; Miwa et al., 2007; Nakagawa et al., 2007; Tanaka et al., 2008). Takano et al. (2006) found *NIP5;1* is a gene up-regulated under B limitation in *A. thaliana* roots. As described in Introduction, NIP5;1 is localized to the plasma membrane and shown to be as a boric acid channel to facilitate B across the membrane. In 2008, Tanaka et al. revealed that NIP6;1 is also a boric acid channel required for proper distribution of boric acid and involved in xylem-phloem transfer of boric acid at the nodal regions. In contrast to *NIP5;1*, the transcript accumulation of *NIP6;1* is elevated in young rosette leaves and shoot apices but not in roots (Tanaka et al., 2008).

To investigate B deficiency responsive genes, similar to the case of *NIP5;1*, I searched for genes up-regulated under B-deficient conditions in *A. thaliana* roots using a combination of DNA microarray and quantitative real-time PCR analysis. Since DNA microarray has already been done by Dr. M. Tanaka, I used the microarray data and selected 16 genes and examined the expression levels using quantitative real-time PCR. In this study, I focused on *GLIP1*. The physiological functions of *GLIP1* were examined and discussed the possible roles of *GLIP1* gene responsive to B deficiency in *A. thaliana*.

4.2 Materials and Methods

4.2.1 Plant materials

Col-0 (ecotype Columbia) of *Arabidopsis thaliana* (L.) Heynh. was from our laboratory stock. Two independent mutant alleles for *GLIP1* T-DNA insertion lines, SALK_130146 and SALK_119002, in the Col-0 background were obtained from ABRC. Homozygous lines for each T-DNA insertion were selected by PCR analysis using primer specific to genome DNA and T-DNA. The positions of the T-DNA insertion sites were then verified by nucleotide sequence determination. Primers used are shown in Table 4-1.

Table 4-1. Genes and gene-specific primers used for homozygous T-DNA insertion line selection and nucleotide sequence determination.

T-DNA insertion line	Oligonucleotide primer sequences, 5´to 3´	
	Forward	Reverse
T-DNA	GCGTGGACCGCTTGCTGC AACT	TGACAGGATATATTGGCGGGTAAAC
SALK_130146	CCTCC TTGAACCCTTCAAAAC	TTGTAACAAACCAATCCGCTC
SALK_119002	GACCAGCAGCTACATTAAGCG	CAACGAGAAGCT TCTGAATGG

4.2.2 Plant growth conditions

For phenotypic analysis, determination of *GLIP1* mRNA accumulation, and determination of B concentration, plants were grown on the MGRL solid medium (Fujiwara et al., 1992). B concentrations were adjusted with boric acid without the change in pH. Two% sucrose and 1.5% gellan gum were added to make the solid media.

Surface-sterilized seeds were sown on the plates and incubated for one day at 4°C. The plates were placed vertically and incubated at 22°C under a 16-h light/8-h dark cycle.

For phenotypic analysis of *GLIP1* T-DNA insertion mutants under various nutrient conditions, plant were grown on MGRL medium containing 1% sucrose solidified with 1% agar without B, Fe, Ca, Cu, Mo, Mg, Mn, Zn, N, P or K. After one day of incubation at 4 ºC, the plates were placed vertically in a growth chamber (16-h light/8-h dark cycle, 22 ºC) for 14 d.

4.2.3 Quantification of transcript accumulation by real-time PCR

Plants were grown under long-day conditions in solid medium supplied with 0.03, 0.1, 0.3 or 100 µM B for 14 days. Roots were separately collected from three independent plants. Total RNA was extracted using the RNeasy Plant Mini Kit as described by the manufacturer. Total RNA (500ng) was reverse-transcribed into cDNA in a 20 µl reaction using the PrimeScript RT reagent kit with oligo$(dT)_{16}$ primer for RT-PCR. The cDNA was amplified by PCR in a Thermal Cycler Dice with SYBR Premix Ex TaqII. The *Elongation Factor1-α* (*EF1-α*) gene was used as a control for quantitation. The primers used in real-time PCR were as follows: 5´-ATTCAAATACGCCCTTCACG-3´ and 5´-ACTTTGCGACAGTCCCATT C-3´ for *GLIP1*; 5-CCTTGGTGTCAAGCAGATGA-3´ and 5´-TGAAGACACCTCCTTGATG ATTT-3´ for *EF1-α.* Specific amplification of target genes was confirmed by electrophoresis. The relative expression level of *GLIP1* was calculated using the standard curve method and standardized using *EF1-α* as a calibrator.

4.2.4 Measurement of B and Ca concentration in plants

To determine the B concentration in roots and shoots of Col-0 and T-DNA mutant lines, plants were grown in solid medium containing 0.03, 0.1, 0.3 or 100 μM B for 14 d under long-day conditions. Roots and shoots were separately harvested from three independent plants and dried in oven at 60°C for more than 60 h. Dry weight was determined and concentrated HNO_3 was added followed by the complete digestion at 130ºC for more than 2 h in a teflon tube until the residue was completely dried. Two ml of 0.08N HNO_3 containing 5 ppb Be was added to dissolve the pellet. The B contents of these samples were determined using ICP-MS.

To determine the Ca concentration in roots and shoots of Col-0 and *GLIP1* T-DNA mutant lines, plants were grown in solid medium containing 0.2 or 2mM Ca for 14 d under long-day conditions. Ca concentrations were determined by ICP-MS as described in B determination.

4.3 Results

4.3.1 Identification of *A. thaliana GLIP1* as a gene up-regulated under B limitation in roots

To search for genes that response to B deficiency, microarray analysis was carried out using *A. thaliana* roots under B deficient condition. The microarray analysis was performed by Dr. Tanaka and found that 88 candidate genes were up-regulated by B deficiency in *A. thaliana* roots (Appendix 2). The expression levels of 16 candidate genes are up-regulated more than 2.1 folds under B deficiency by using quantitative real-time PCR. Among 16 genes, 5 genes, including *GLIP1*, *FAMT*, *F4P12*, *CYP71A12* and *MDC8*, were up-regulated in B-deficient roots as compared with B-sufficient roots (Figure 4-1). Among the 5 genes, *GLIP1* (At5g40990) was selected for further analysis on the phenotype of the mutant plants and studied in its function as described below.

The expression levels of *GLIP1* in response to B deficiency was analysed by quantitative real-time PCR. These experiments were repeated two times, and similar results were obtained in each case. The results suggested that the expression levels of *GLIP1* transcript was up-regulated under low B condition. The mRNA levels of *GLIP1* under low B condition was 25.1 times higher than under sufficient condition (Figure 4-2).

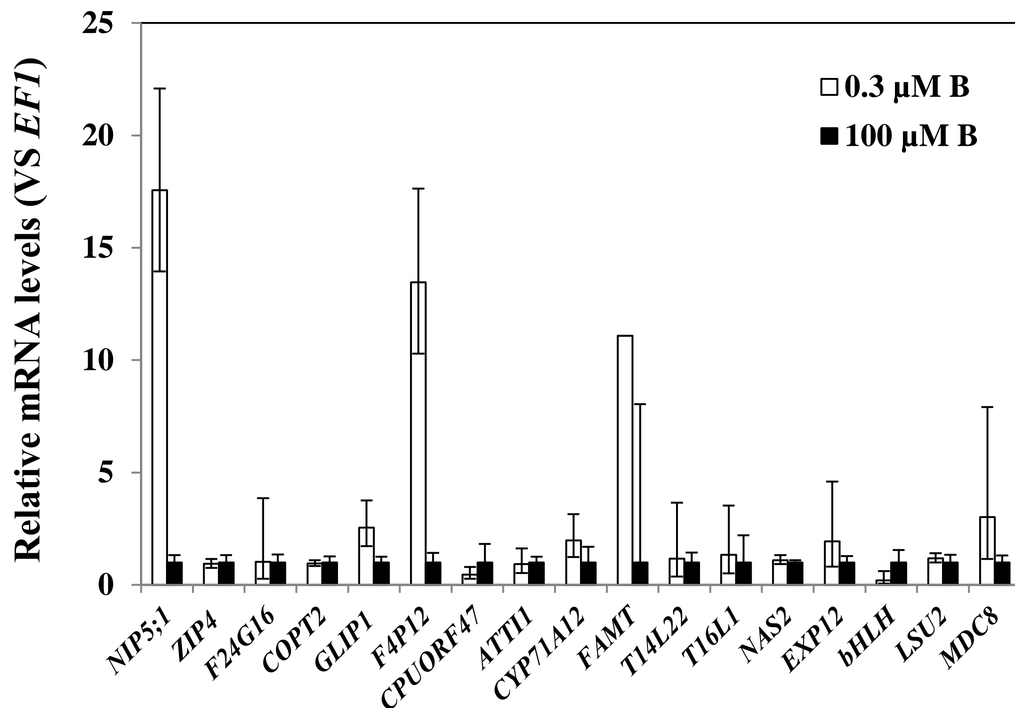


Figure 4-1. Relative mRNA levels of the 16 candidate genes that were up-regulated under B deficiency from microarray data were verified by using quantitative real-time PCR in roots of Col-0 plants. Plants were grown on solid medium containing 0.3 or 100 μM boric acid. The transcript levels of each gene at 100 μM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3).

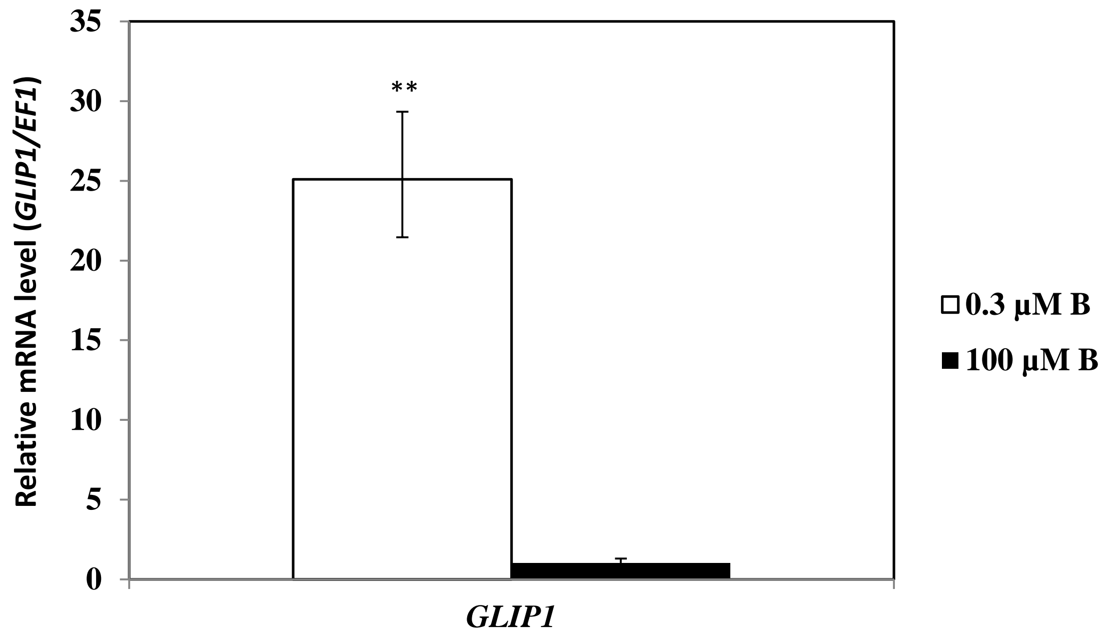


Figure 4-2. Accumulation of *GLIP1* (At5g40990) mRNA in roots of Col-0 plants. Plants were grown on solid medium containing 0.3 (B-) or 100 (B+) µM boric acid. The transcript levels of *GLIP1* at 100 µM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). Statistical significance was determined using Student's t-test; ** $P < 0.01$.

4.3.2 Isolation of *GLIP1* T-DNA insertion mutants

To investigate the function of the *GLIP1* gene in *A. thaliana*, two independent mutant alleles for *GLIP1* were obtained. SALK_130146 and SALK_119002, T-DNA insertion lines of *GLIP1*, were obtained from ABRC and named *glip1-2* and *glip1-3*, respectively. Homozygous lines for each T-DNA insertion were selected by PCR analysis (Figure 4-3A).

The exact position of T-DNA insertion lines were verified by the nucleotide sequence analysis. T-DNA was inserted in the fourth exon and the third exon in *glip1-2* and *glip1-3*, respectively (Figure 4-3).

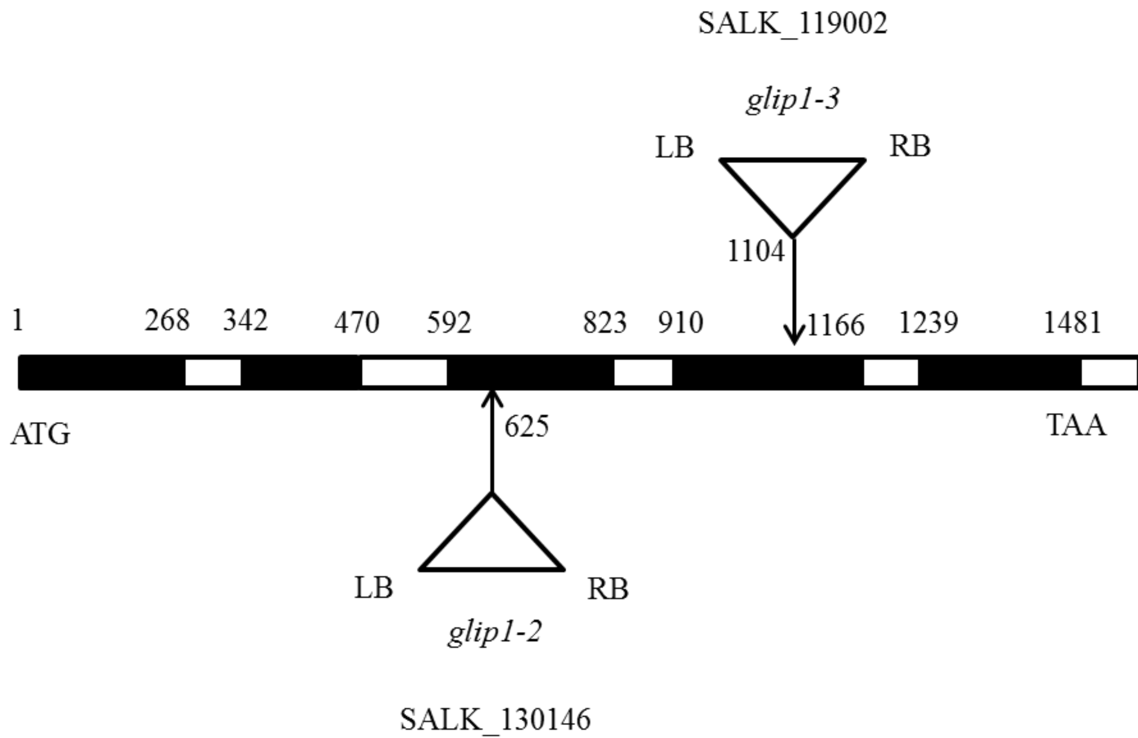


Figure 4-3. *GLIP1* mutant alleles. Schematic representation of a T-DNA insertion in the *GLIP1* gene and the exon-intron structure of the genes. Black and white bars indicate exons and introns, respectively.

4.3.3 Expression and functional analysis of *GLIP1*

4.3.3.1 Phenotypic analysis of *GLIP1* T-DNA insertion mutants

To characterize the function of *GLIP1* gene in *A. thaliana*, two independent T-DNA insertion mutant lines for *GLIP1*, *glip1-2* and *glip1-3*, were used. The T-DNA insertion lines and wild-type (Col-0) plants were grown under 0.03, 0.1, 0.3 or 100 µM B for 14 days. Both T-DNA insertion lines showed normal root growth as compared with wild-type plants at 0.1, 0.3 or 100 µM B conditions. Whereas at 0.03 µM B, the root elongation rate of both lines, especially the root growth rate was reduced and shorter than wild-type at 10 days after germination (Figure 4-4). No difference between *glip1-2* and *glip1-3* was observed in sensitivity to B deficiency. Taken together, these results suggest that root growth of *glip1* may sensitive to B deficient conditions.

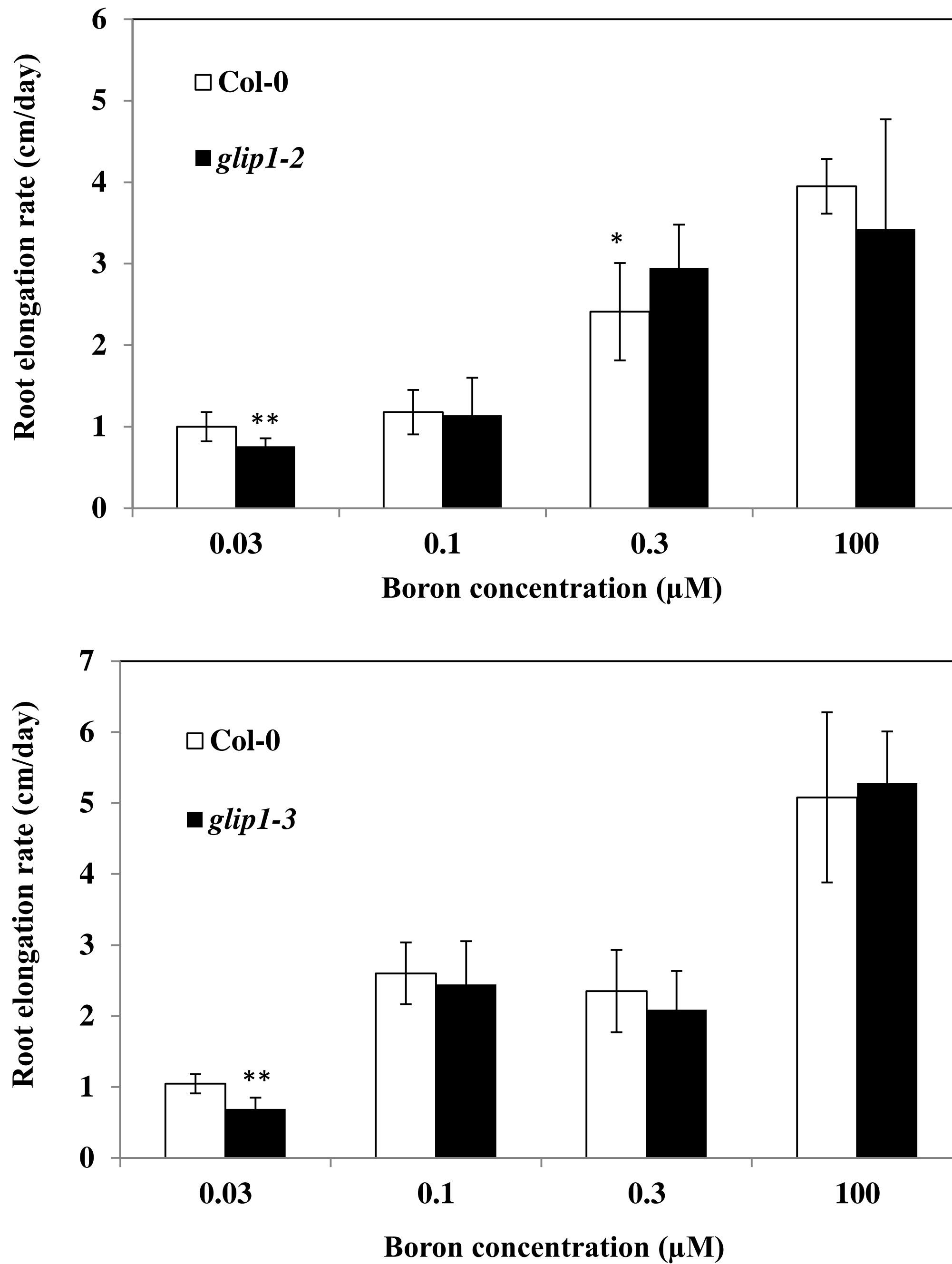
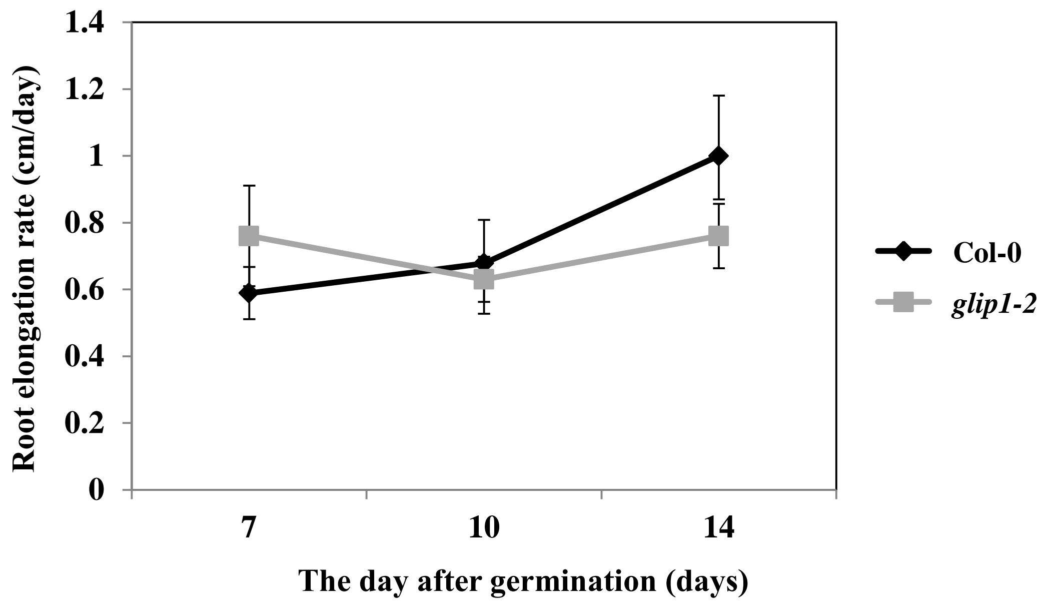


Figure 4-4. Root elongation rate of *GLIP1* T-DNA lines under 0.03, 0.1, 0.3 or 100 µM B at 10-14 days after germination. Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)



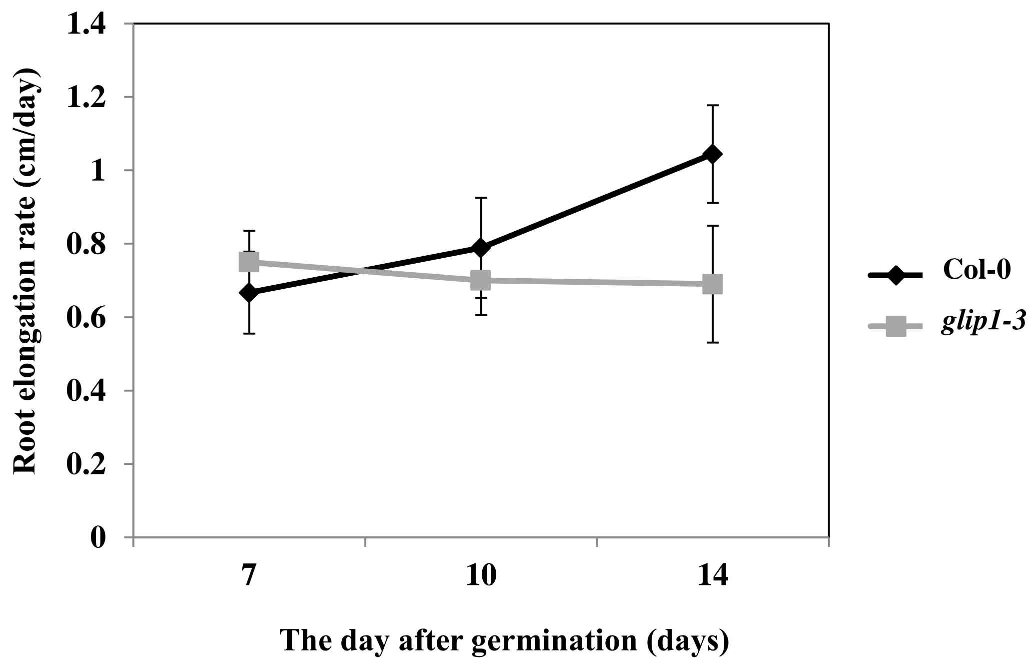


Figure 4-5. Root elongation rate of *GLIP1* T-DNA lines and Col-0 plants after germination 7, 10 or 14 days under 0.03 μM B. Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

4.3.3.2 B concentration in *GLIP1* T-DNA insertion plants

To examine the physiological function of *GLIP1*, the B concentrations of the T-DNA lines and wild-type plants grown under various B concentrations (0.03, 0.1, 0.3 or 100 μM B) were determined using ICP-MS. There were no difference in the B concentrations in roots and shoot between Col-0 and *glip1* T-DNA plants, except at 100 μM B condition in shoot. The B concentrations of both *glip1* plants were significantly reduced (Figure 4-6).

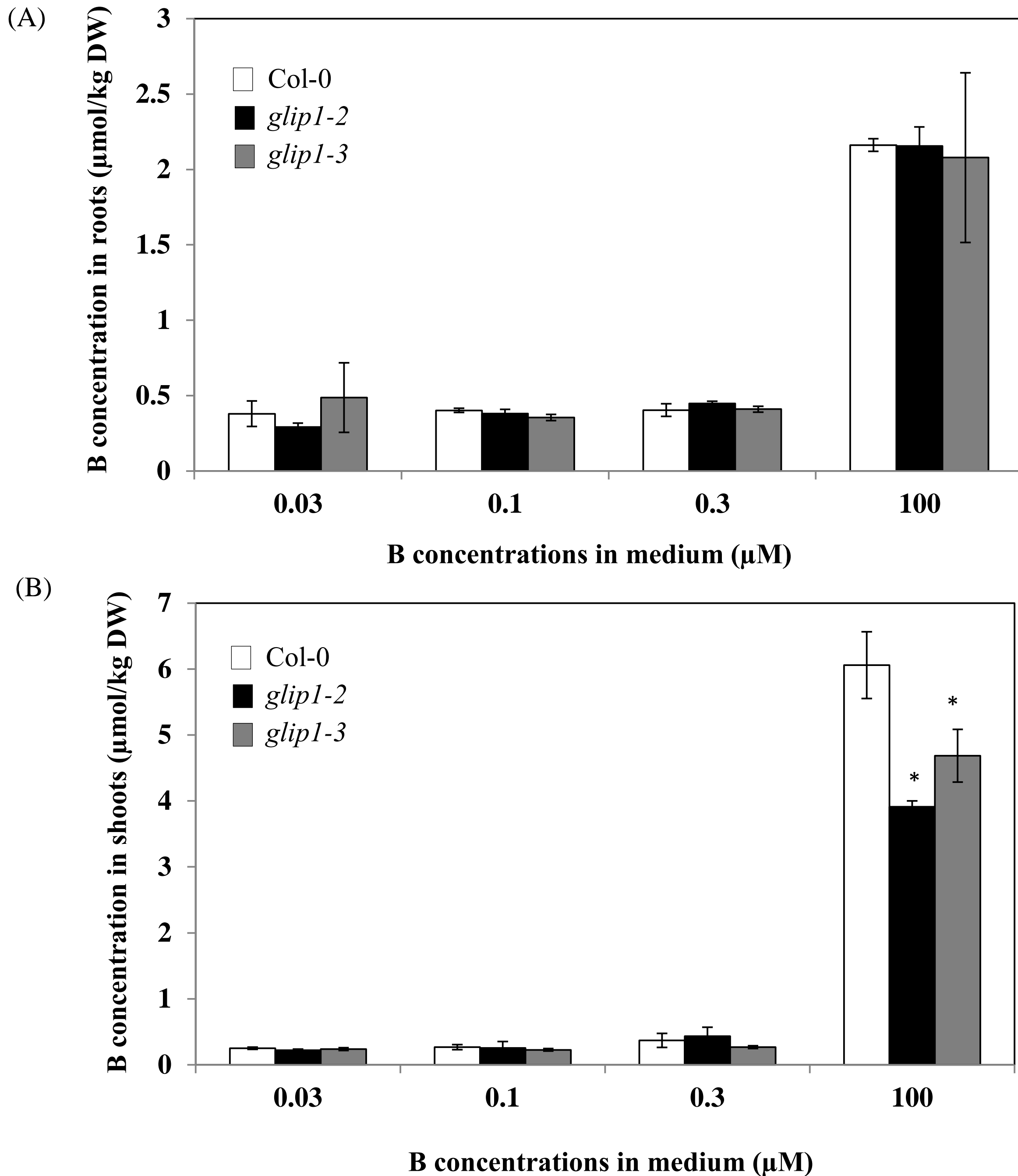


Figure 4-6. The B concentrations in roots (A) and shoots (B) of Col-0 and *GLIP1* T-DNA lines for *GLIP1*. Plants were grown in solid medium containing 100, 0.3, 0.1 or 0.03 µM boric acid for 14 days. Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

4.3.3.3 Phenotypic analysis of *GLIP1* T-DNA insertion mutants under various nutrient conditions

To demonstrate whether the mutant phenotype is specific to B deficiency, the phenotype of the T-DNA insertion lines and Col-0 plants were examined on the medium without boron (B-), iron (Fe-), calcium (Ca-), copper (Cu-), molybdenum (Mo-), magnesium (Mg-), manganese (Mn-), zinc (Z-), nitrogen (N-), phosphate (P-), or potassium (K-). The results showed that there were no significantly difference in root elongation between the mutant lines and wild type plants under all of the nutrient deficient conditions (Figure 4-7A). While, the shoot weight of both *GLIP1* mutant lines was significantly reduced in Ca deficient condition compared with wild type plants (Figure 4-7B).

To confirm the phenotype of *GLIP1* T-DNA insertion lines under Ca deficiency, the mutants and Col-0 plants were grown under 0, 0.15, 0.2, 0.25 or 2 mM Ca conditions. Under low Ca conditions (0, 0.15, 0.2, and 0.25 mM Ca), root length was not statistically different between the Col-0 and mutants (Figure 4-8A). In contrast, shoot fresh weight of both *GLIP1* mutant lines was significantly reduced under 0, 0.15, 0.2, and 0.25 mM Ca conditions compared with Col-0, except for 2 mM Ca condition, indicating that the shoot is sensitive to Ca deficiency (Figure 4-8B and 4-9).

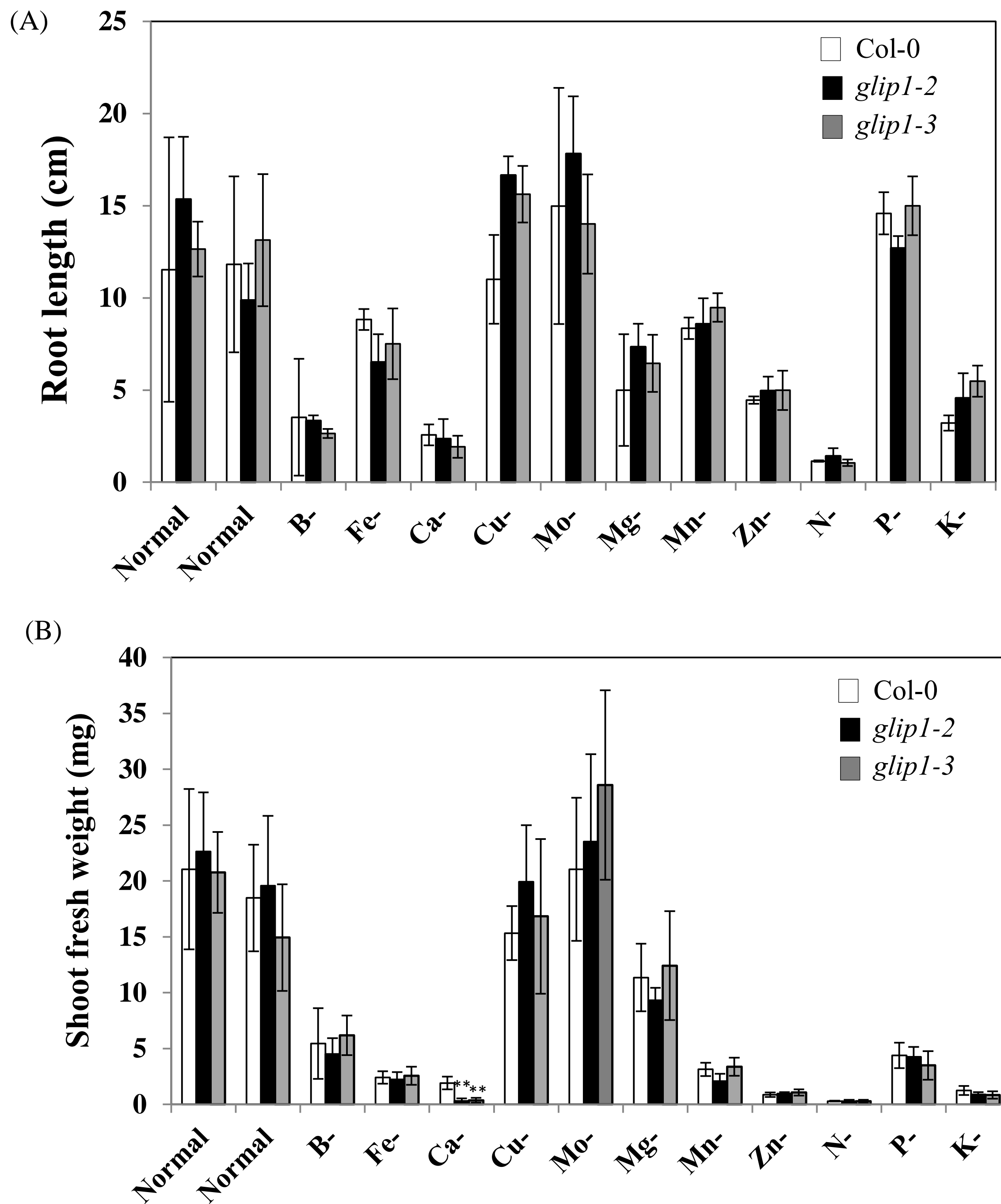
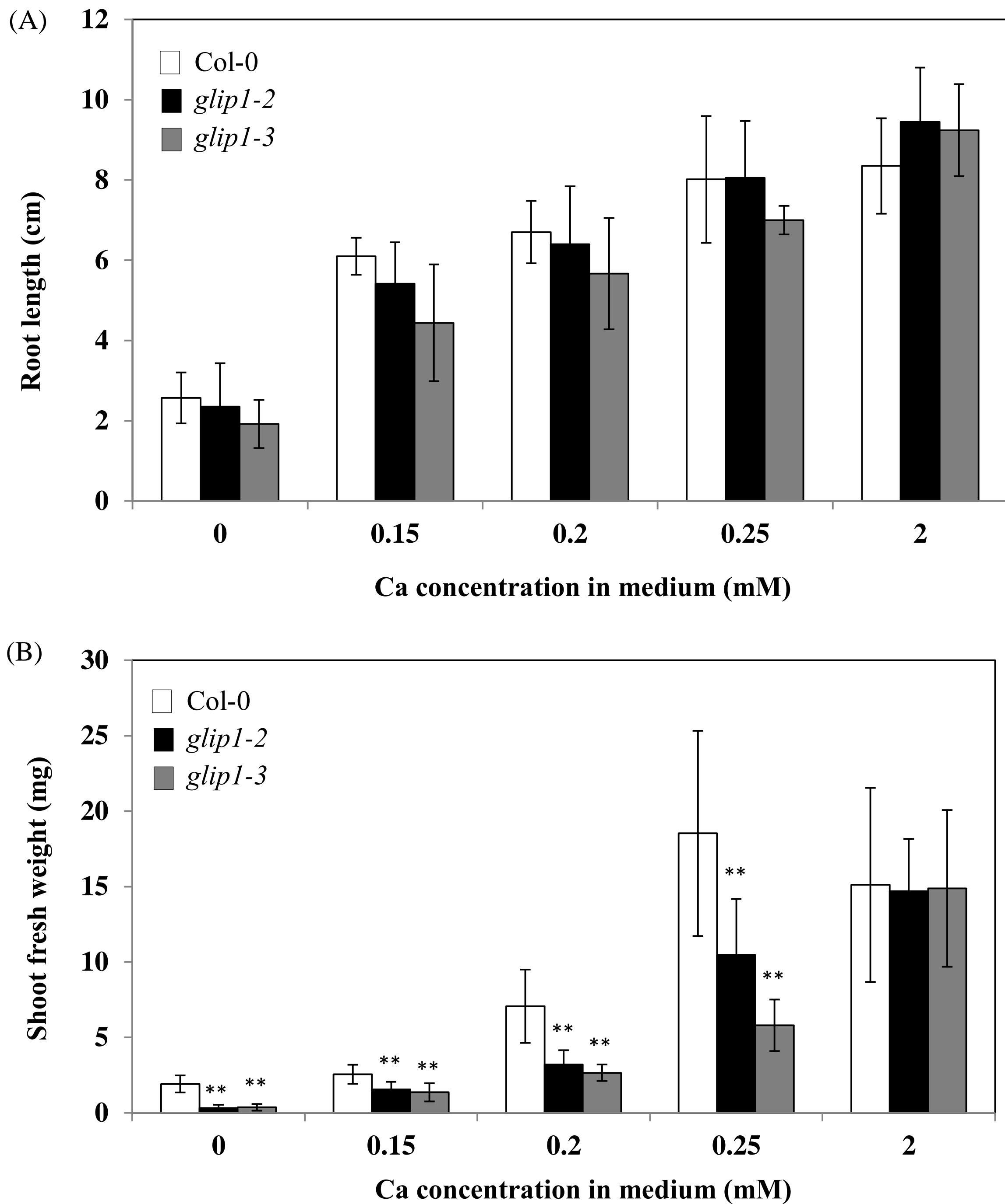


Figure 4-7. Root length (A) and shoot fresh weight (B) of Col-0 and *GLIP1* T-DNA lines under nutrient deficient conditions after germination 14 days (n=5-10); * $P < 0.05$, ** $P < 0.01$ by Student's T-test (B; boron, Fe; I ron, Ca; calcium, Cu; copper, Mo; molybdenum, Mg; magnesium, Mn; manganese, Zn; zinc, N; nitrogen, P; phosphorus, K; Potassium)



Figur4-8. Root elongation (A) and shoot fresh weight (B) of *GLIP1* T-DNA lines under 2, 0.25, 0.2, 0.15 or 0 mM calcium conditions after germination 14 days (n=5-10) Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

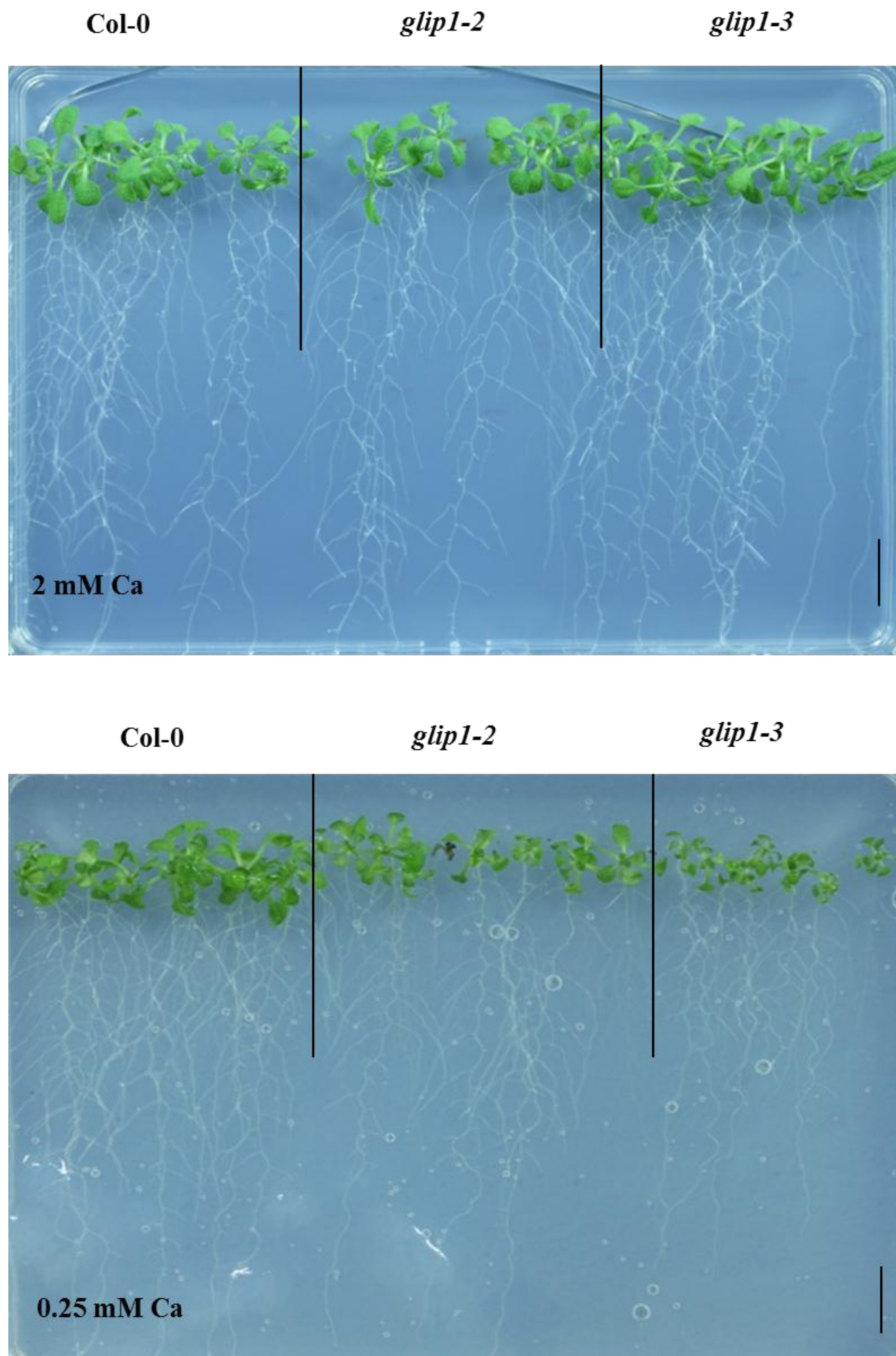
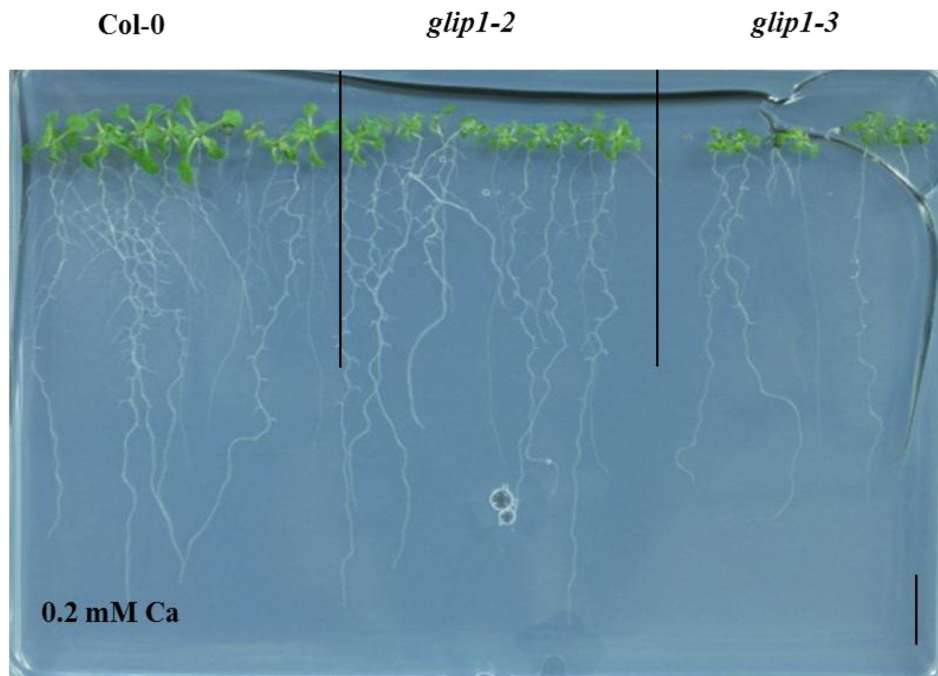


Figure 4-9. Plants were grown in solid medium containing 2, 0.25, 0.20, 0.15 or 0 mM Ca for 14 days. Picture of Figure 4-8. Bars show 1 cm. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)



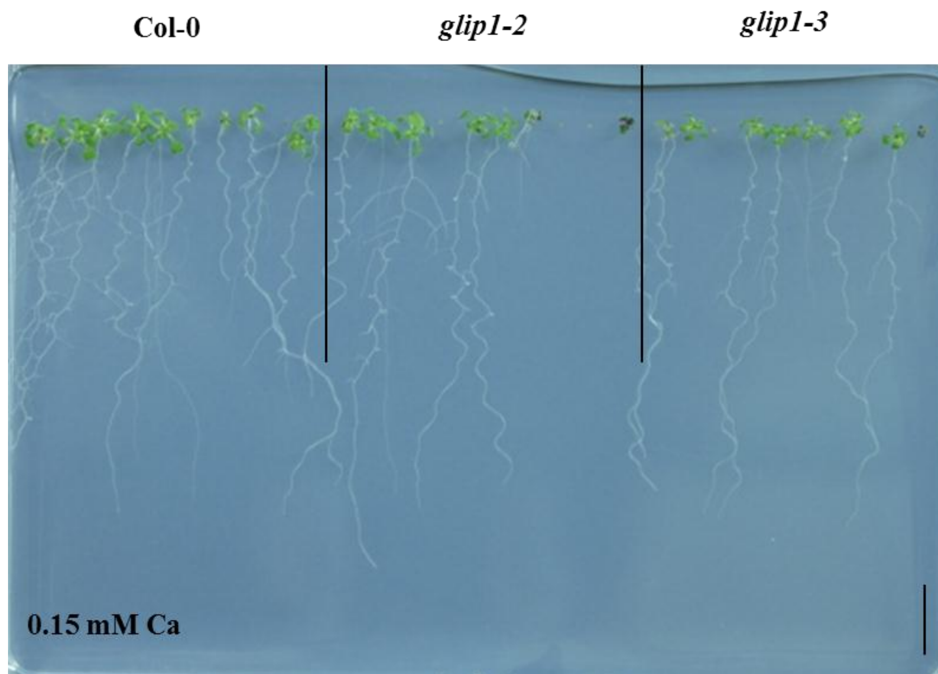


Figure 4-9 (Continue). Plants were grown in solid medium containing 2, 0.25, 0.20, 0.15 or 0 mM Ca for 14 days. Picture of Figure 4-8. Bars show 1 cm. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

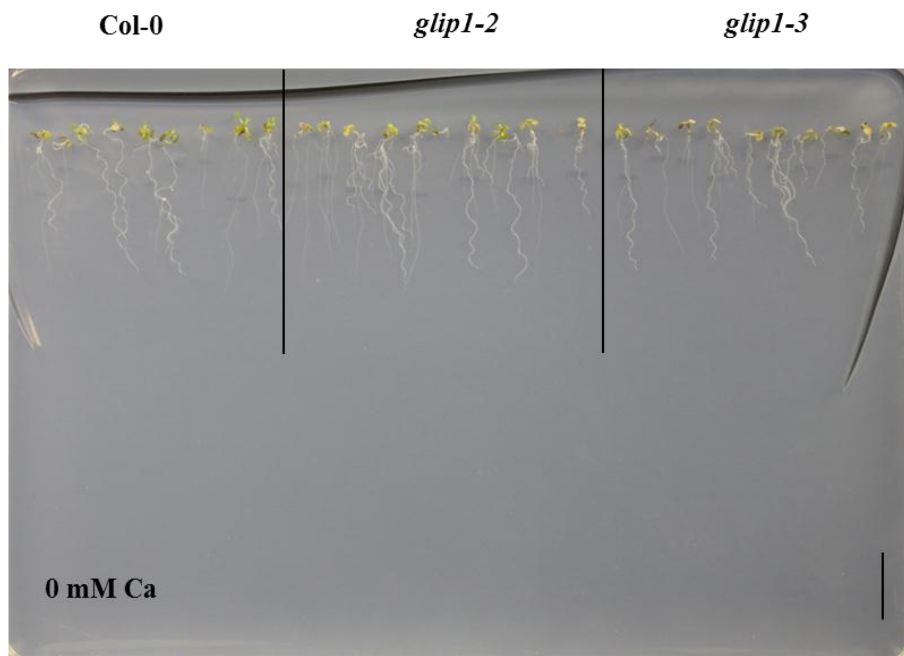


Figure 4-9 (Continue). Plants were grown in solid medium containing 2, 0.25, 0.20, 0.15 or 0 mM Ca for 14 days. Picture of Figure 4-8. Bars show 1 cm. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

4.3.3.4 *GLIP1* transcript accumulation in T-DNA insertion lines

Accumulation of *GLIP1* mRNA in the insertion lines and the wild-type plants was quantified by quantitative real-time PCR. Plants were grown on solid medium containing 0.2 or 2 mM Ca for 14 d. The *GLIP1* mRNA levels in roots of *glip1-2* mutant plants were 10 and 17.6% of those of the Col-0 plants at 0.2 and 2 mM Ca, respectively (Figure 4-10A). The mRNA levels in roots of *glip1-3* mutant plants were 32 and 12 % of those of Col-0 plants at 0.2 and 2 mM Ca, respectively. In shoots, *GLIP1* mRNA level in *glip1-3* was higher at 0.2 mM Ca compared with Col-0 plants (Figure 4-10).

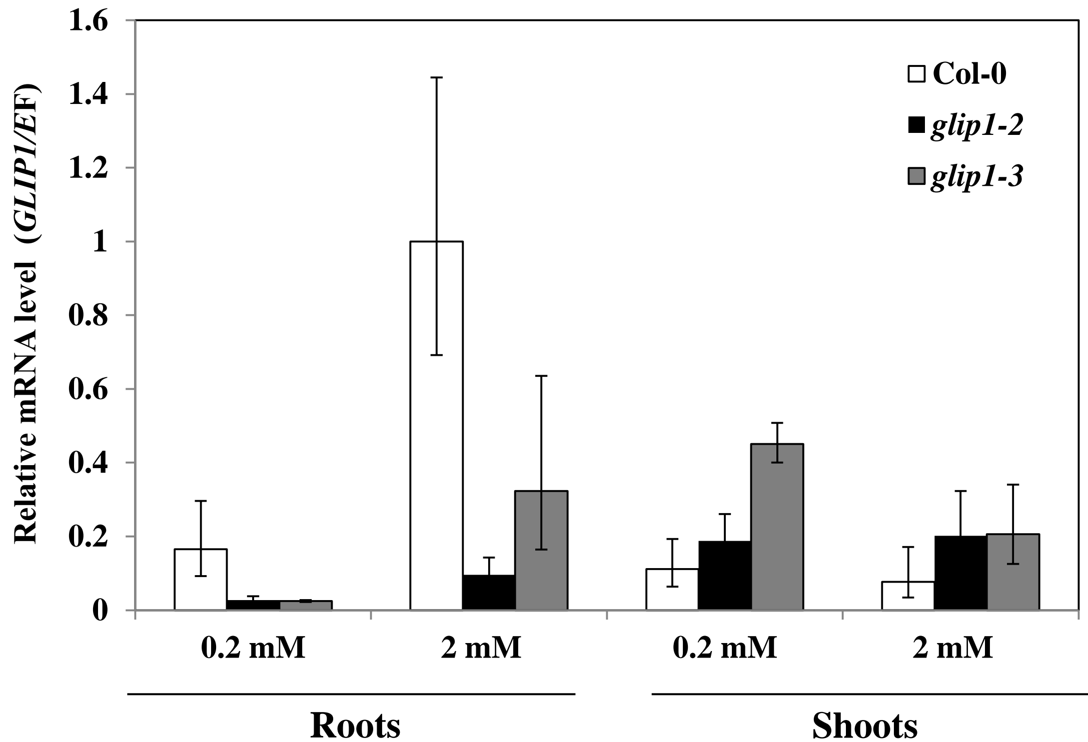
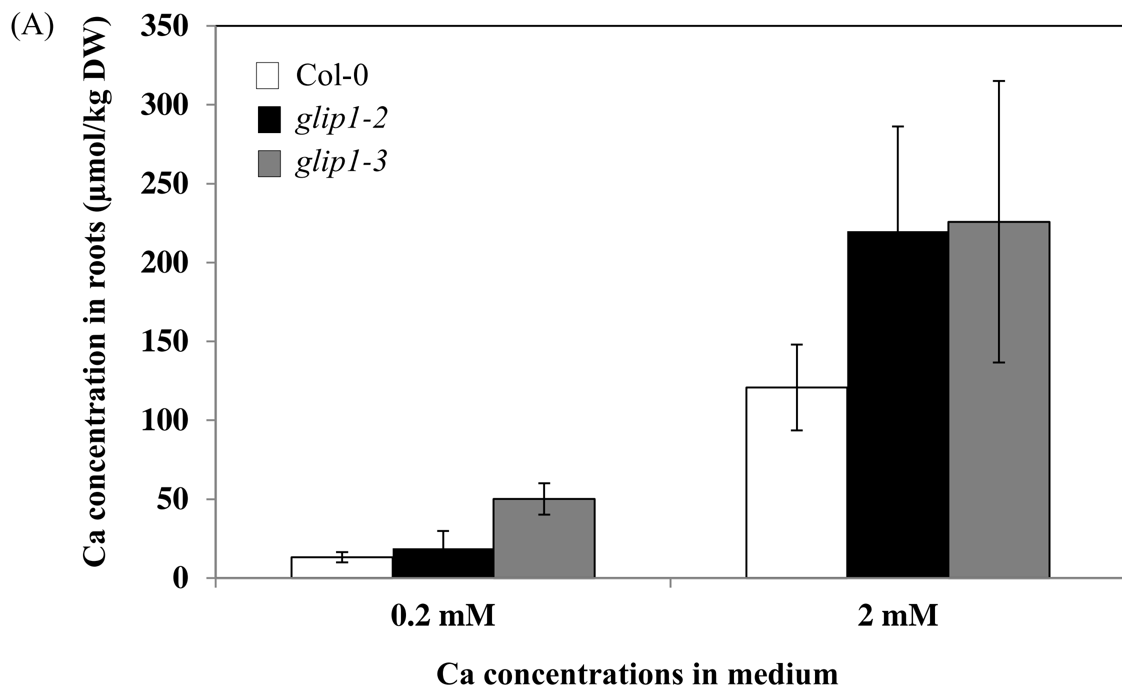


Figure 4-10. Accumulation of *GLIP1* mRNA in roots and shoots of Col-0 and *GLIP1* insertion lines. Plants were grown on solid medium containing 0.2 or 2mM Ca for 14 days. The transcript levels of *GLIP1* in Col-0 plants at 100 μM B was used as the control and assigned value of 1. Means of three biological replicates±SE for each treatment are shown (n=3). (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

4.3.3.5 Ca concentration in *GLIP1* T-DNA insertion plants

To further elucidate the functions of *GLIP1* in Ca uptake and transport, the Ca concentrations in the roots and shoots of Col-0 and mutant plants grown under 0.2 or 2 mM Ca conditions were determined using ICP-MS. Roots and shoots were collected from three biological replicates. Roots of *glip1-2*, *glip1-3* and Col-0 plants showed no significant difference in Ca concentrations when grown in medium containing 0.2 or 2 mM Ca. The significant reductions in Ca concentrations were observed in *glip1-2* and *glip1-3* in shoots under 0.2 mM Ca. In shoots under 2 mM Ca, the Ca concentrations in shoots of *glip1-2* and *glip1-3* were no significant difference as compared with Col-0 plants (Figure 4-11), suggesting that *GLIP1* may involve in Ca uptake and transport from roots to shoots under low Ca condition .



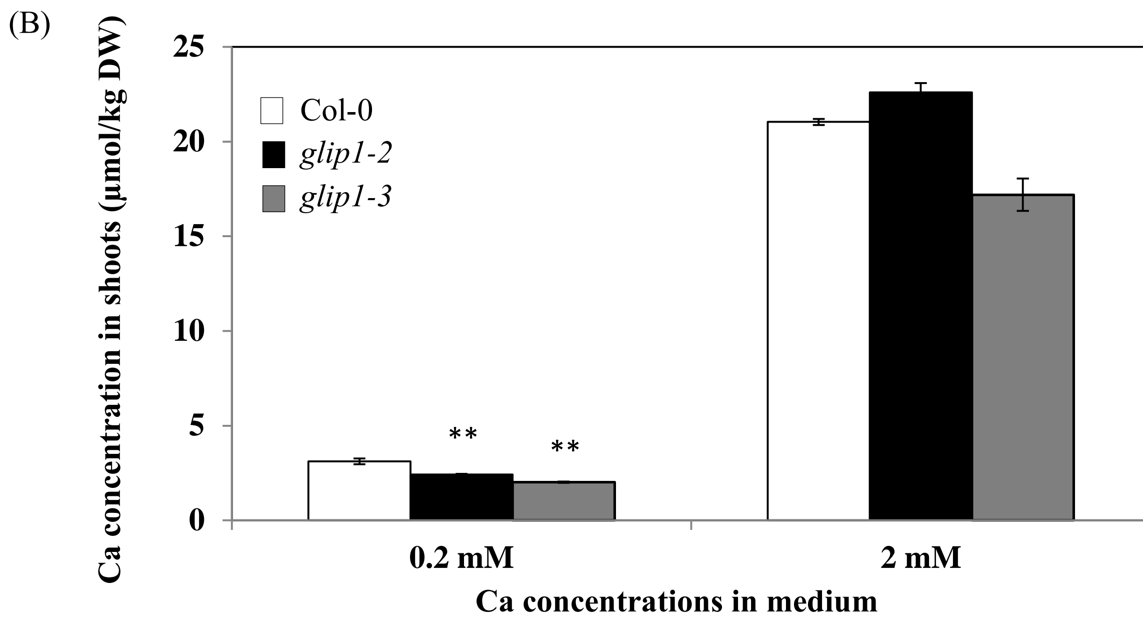


Figure 4-11. The Ca concentrations in roots (A) and shoots (B) of Col-0 and *GLIP1* T-DNA lines. Plants were grown in solid medium containing 0.2 or 2mM Ca for 14 days. Statistical significance was determined using Student's t-test; * $P< 0.05$, ** $P< 0.01$. (*glip1-2*, SALK_130146; *glip1-3*, SALK_119002)

4.4 Discussion

4.4.1 *GLIP1* is induced by B deficiency

It has been reported that B deficiency induces the expression of stress responsive genes (Gonzalez-Fontes et al., 2008). In this study, the *GLIP1* gene (At5g40990) was identified from *A. thaliana* roots as a gene whose expression is significantly increased under B deficient condition (Figure 4-1 and 4-2). *GLIP1* is GDSL lipase that perform critical roles in the biotic and abiotic stress responses (Lee et al., 2009). Physiologically, plant GDSL lipases are generally considered to be mainly involved in the regulation of plant growth and development (Ling, 2008). GLIP1 is localized in the intercellular space and is secreted into the cell wall or extracellular space (Oh et al., 2005). Alves et al. (2010) observed that B deficiency altered the expression of gene related with lipid transport-related process and lipid metabolic processing.

4.4.2 A possible role of *GLIP1* in plant growth under B or Ca deficiency

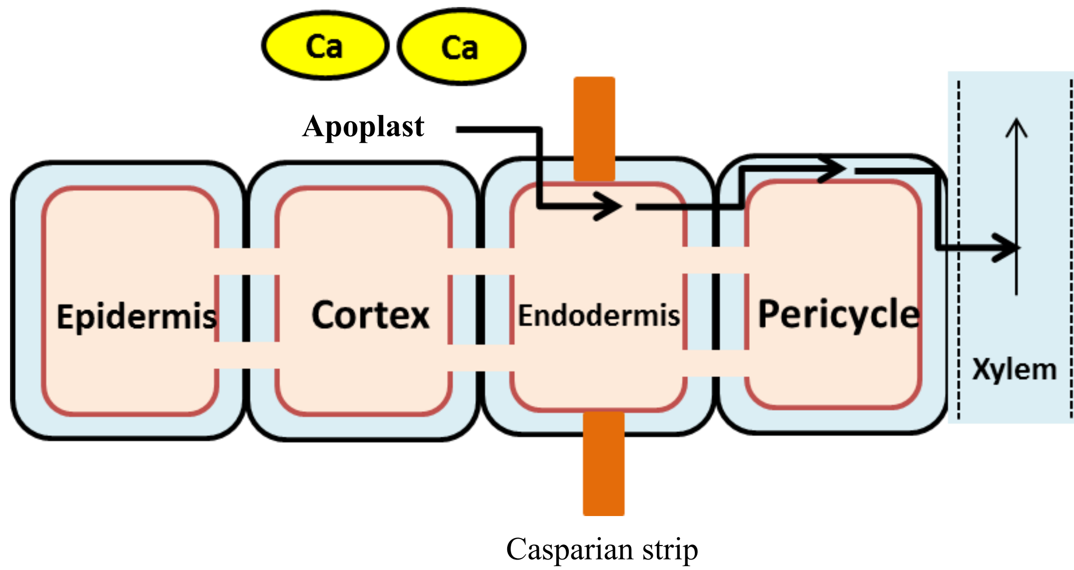
Under Ca or B limitation, both *glip1-2* and *glip1-3* were affected the phenotype (Figure 4-4 and 4-8). In addition, the Ca concentrations in shoots of both insertion plants were reduced (Figure 4-11). These data indicate that GLIP1 is important for Ca transport from roots to shoots under low Ca condition.

GLIP1 protein has a lipase activity that catalyses the hydrolysis of a lipid or phospholipid. GLIP1 also exhibit esterase activity (Oh et al., 2005). It is localized in the apoplastic space. In the Ca transport pathway, Ca^{2+} ion move across the plasma membranes of endodermal cells by apoplastic pathway (Figure 4-12). In *A. thaliana*,

suberin act as apoplastic barriers. Its function is to prevent water and nutrients taken up by the root from entering the stele through the apoplast. Suberin consists of two domains, including a polyaromatic and a polyaliphatic domain. The aliphatic domain of suberin is a polyester polymer (Franke et al., 2005). Taken altogether, it is possible that suberin may be degraded by GLIP1. So, the loss of GLIP1 in the mutants may accumulate the suberin and result in low Ca in shoots. While, in roots, plant can directly get Ca from the medium, Ca may be enough for root growth.

In case of B, B is transported through both symplast and apoplast, considering that is different from Ca transport (Figure 4-12). Although, suberin were accumulate and affect the apoplastic pathway of B transport, B still has enough for shoot growth of the mutant under low B condition. Because B can uptake and transport through symplastic pathway by NIP5;1. For root phenotype of the mutants, now it is still unclear how GLIP1 affect the root length under low B conditions. However this hypothesis can explain most occurrences.

(A) Ca transport pathway



(B) B transport pathway

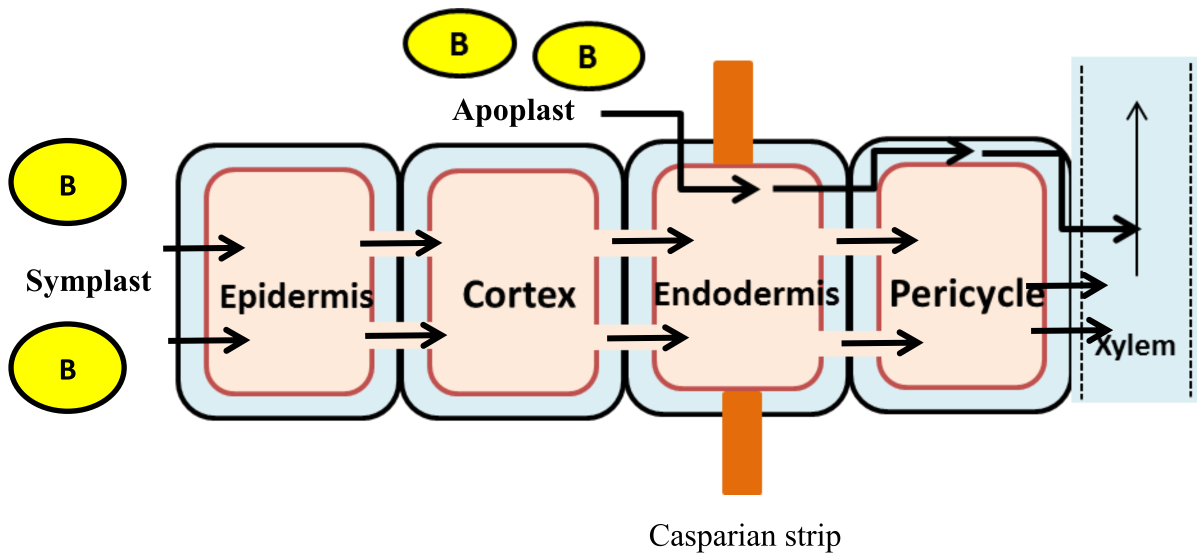


Figure 4-12. The models of Ca transport (A) and B transport (B) in *A. thaliana* roots.

Conclusion

The aim of this study was to: (I) isolate novel B transporter gene in hexaploid wheat and characterize the function of these gene, (II) comparison the expression of genes related to known B efflux transporter in two genotypes with different B efficiency in cereal plants (wheat, maize and rice) in Thailand, (III) identify gene that response to B deficient condition.

Three genes closely related to Os *BOR1* were cloned from wheat, named Ta *BOR1.1*, Ta *BOR1.2* and Ta *BOR1.3*. The Ta *BOR1s* genes are efflux-type B transporters that localize to the plasma membrane. The accumulation of Ta *BOR1s* are regulated at transcriptional level. This is the first identification and characterization of BOR1-like gene in wheat which has complex genomes. It is also the new finding that the regulation of Ta BOR1 is clear contrast with the regulation of At BOR1 (Chapter 2).

The mechanism in tolerance to B deficiency is probably regulated by the expression level and/or function of the B transporter. The expression patterns of *BOR1*-like genes are differentially among two different genotypes. *BOR1*-like gene expressed at high level in B-efficient compared to B-inefficient genotypes of wheat, maize and rice. It is possible that the expression levels of *BOR1*-like genes correlate with the B deficiency tolerance in plants. Moreover, *BOR1*-like genes can be useful as gene expression biomarkers for crop breeding in wheat maize and rice by selecting appropriate tissues and growth stages (Chapter 3).

The *GLIP1* gene (At5g40990) was identified as a gene whose expression is increased significantly under B deficient condition. *GLIP1* may be required for root

growth of *A. thaliana* plants under B deficient and necessary for uptake and xylem loading of B from roots to shoots and functions under normal B condition. In addition, *GLIP1* mutant plants were significantly reduced shoot weight and Ca concentrations in shoots under Ca deficiency, suggesting that *GLIP1* required for *A. thaliana* shoot growth and involve in the Ca transport from roots to shoots. This is the first report that GDSL lipase relate to nutrient transport pathways in plants (Chapter 4).

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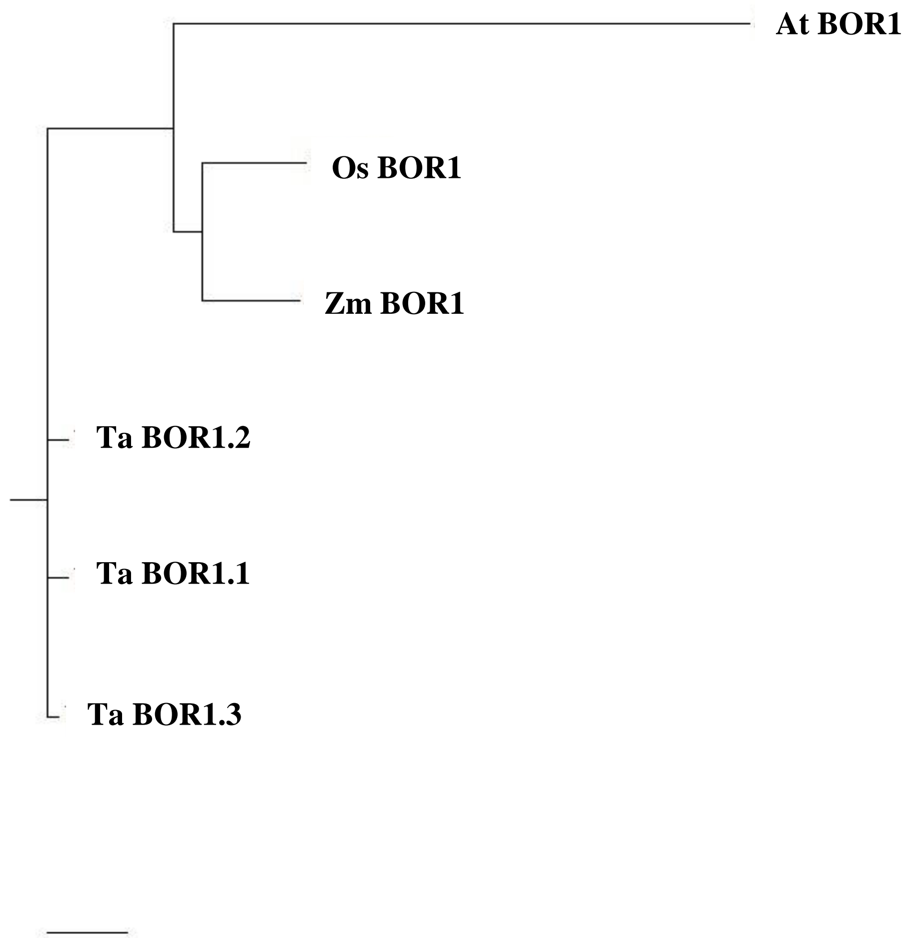

Appendix 1 Phylogenetic relationship of BOR1-like proteins of wheat, *A. thaliana,* maize and rice. Phylogenetic analysis was performed with clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Appendix 2 List of 88 candidate genes differentially expressed between B-sufficient (100 µM B) and deficient (0.3 µM B) conditions in *Arabidopsis* Col-0 roots. These data were obtained by microarray analysis (by Dr. M. Tanaka).

Prob set	AGI code	Gene description	microarray results		
			0.3 µM B	100 µM B	fold change
251438_s_at	At3g59930	defensin-like (DEFL) family protein	21426.5	1475.1	14.5
251937_at	At3g53400	expressed protein	1734.9	163.5	10.6
252502_at	At3g46900	copper transporter family	434.1	45.3	9.6
254971_at	At4g10380	NIP5;1 major intrinsic family protein	7624	875	8.7
249333_at	At5g40990	GDSL-motif lipase/hydrolase family protein	90.6	12.7	7.1
250955_at	At5g03190	CONSERVED PEPTIDE UPSTREAM OPEN READING FRAME 47 expressed protei	510.9	79	6.5
260551_at	At2g43510	trypsin inhibitor	219.4	43.1	5.1
267565_at	At2g30750	cytochrome P450 71A12	321.1	67.9	4.7
254758_at	At4g13260	flavin-containing monooxygenase	86.2	21.8	4.0
260462_at	At1g10970	metal transporter, member of the Zinc (Zn2+)-Iron (Fe2+) permease (ZIP) family	829.1	213.8	3.9
246340_s_at	At3g44860	S-adenosyl-L-methionine:carboxyl methyltransferase family protein	246.7	69.9	3.5
248151_at	At5g54270	chlorophyll A-B binding protein / LHCII type III (LHCB3) identical to Lhcb3 protein	105.3	30.3	3.5
265121_at	At1g62560	flavin-containing monooxygenase family protein	97.8	28.5	3.4
259575_at	At1g35320	expressed protein	81.9	25.5	3.2
266336_at	At2g32270	zinc transporter (ZIP3); member of the Zinc (Zn2+)-Iron (Fe2+) permease (ZIP) family	7159.2	2245.9	3.2

Prob set	AGI code	Gene description	microarray results		
			0.3 μM B	100 μM B	fold change
260703_at	At1g32270	syntaxin, putative similar to syntaxin related protein	33.2	10.9	3.0
257927_at	At3g23240	ethylene-responsive factor 1	86	28.3	3.0
261466_at	At1g07690	hypothetical protein	82	27.3	3.0
262707_at	At1g16290	expressed protein	51.2	17.2	3.0
246375_at	At1g51830	leucine-rich repeat protein kinase	180.4	60.7	3.0
247919_at	At5g57650	eukaryotic translation initiation factor	37.7	12.9	2.9
266179_at	At2g02300	F-box family protein	80.6	28.7	2.8
253301_at	At4g33720	pathogenesis-related protein	482.1	172.3	2.8
254098_at	At4g25100	superoxide dismutase [Fe]	7415.3	2717.5	2.7
263877_at	At2g21780	expressed protein	38.3	14.1	2.7
247204_at	At5g64990	Ras-related GTP-binding protein	25.8	9.6	2.7
247678_at	At5g59520	zinc transporter (ZIP2); member of the Zinc (Zn2+)-Iron (Fe2+) permease (ZIP) family	2855.7	1065.8	2.7
245875_at	At1g26240	proline-rich extensin-like family protein	53.6	20.3	2.6
257027_at	At3g19210	DNA repair protein RAD54	57.4	22	2.6
248048_at	At5g56080	nicotianamine synthase	2026.5	778.3	2.6

Prob set	AGI code	Gene description	microarray results		
			0.3 μM B	100 μM B	fold change
253347_at	At4g33610	glycine-rich protein	76.4	29.4	2.6
258388_at	At3g15370	expansin (EXP12)	177.2	68.2	2.6
245038_at	At2g26560	patatin	494.6	191.2	2.6
259837_at	At1g52180	major intrinsic family protein	108.9	42.1	2.6
258277_at	At3g26830	cytochrome P450 71B15	271.5	105.1	2.6
261009_at	At1g26360	hydrolase, alpha/beta fold family	97.4	39.2	2.5
250172_at	At5g14330	expressed protein	85.8	34.7	2.5
244957_at	orf157	orf157 hypothetical protein	29.6	12.5	2.4
256208_at	At1g50930	hypothetical protein	84.4	35.9	2.4
247755_at	At5g59090	subtilase family protein	7029.1	3038.2	2.3
259269_at	At3g01270	pectate lyase family protein	30.3	13.1	2.3
264574_at	At1g05300	metal transporter (ZIP5); member of the Zinc (Zn2+)-Iron (Fe2+) permease (ZIP) family	1042.4	452.8	2.3
263558_at	At2g16380	SEC14 cytosolic factor family protein	77.5	34.1	2.3
253413_at	At4g33020	metal transporter (ZIP9); member of the Zinc (Zn2+)-Iron (Fe2+) permease (ZIP) family	248.8	109.5	2.3
252269_at	At3g49580	expressed protein	72.2	31.8	2.3

Prob set	AGI code	Gene description	microarray results		
			0.3 μM B	100 μM B	fold change
257424_at	At1g78840	F-box family protein	36.7	16.2	2.3
262012_s_at	At1g35625	protease-associated zinc finger (C3HC4-type RING finger) family protein	111.6	49.4	2.3
267502_at	At2g45550	cytochrome P450 family protein	58.9	26.2	2.2
260438_at	At1g68290	bifunctional nuclease	56.3	25.2	2.2
252429_at	At3g47500	Dof-type zinc finger domain	123.1	55.3	2.2
258395_at	At3g15500	no apical meristem (NAM) family protein	205.5	92.4	2.2
258851_at	At3g03190	glutathione S-transferase	1374.9	619	2.2
248667_at	At5g48710	ubiquitin-related similar to SP|O13351 Ubiquitin-like protein smt3/pmt3	15.2	6.9	2.2
248725_at	At5g47980	transferase family protein	1107.7	503.1	2.2
244953_s_at	nad6	nad6 NADH dehydrogenase subunit 6	33.9	15.5	2.2
259087_at	At3g04980	DNAJ heat shock N-terminal domain-containing protein	58.3	26.7	2.2
246013_at	At5g10660	calmodulin-binding protein	23.7	10.9	2.2
254326_at	At4g22610	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	652	300.7	2.2
249205_at	At5g42600	pentacyclic triterpene synthase	1885.4	870.2	2.2
249676_at	At5g35960	protein kinase	64.3	29.7	2.2

Prob set	AGI code	Gene description	microarray results		
			0.3 μM B	100 μM B	fold change
265817_at	At2g18050	histone H1-3 (HIS1-3)	343.2	159.9	2.1
263380_at	At2g40200	basic helix-loop-helix (bHLH) family protein	37.1	17.3	2.1
251524_at	At3g58990	aconitase C-terminal domain	3457.5	1616.1	2.1
252111_at	At3g51570	disease resistance protein (TIR-NBS-LRR class)	26.1	12.2	2.1
257021_at	At3g19710	branched-chain amino acid aminotransferase	9666.5	4577.4	2.1
248729_at	At5g48010	pentacyclic triterpene synthase	2834.9	1343.7	2.1
249752_at	At5g24660	expressed protein	210.5	99.8	2.1
253070_at	At4g37850	basic helix-loop-helix (bHLH) family protein	116.3	55.2	2.1
251191_at	At3g62590	expressed protein	185.2	88.1	2.1
250123_at	At5g16530	auxin efflux carrier family protein	73.2	35.4	2.1
254805_at	At4g12480	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	167	80.9	2.1
264754_at	At1g61400	S-locus protein kinase	45	21.8	2.1
263477_at	At2g31790	UDP-glucoronosyl/UDP-glucosyl transferase family protein	926.6	449	2.1
259632_at	At1g56430	nicotianamine synthase	224.9	109	2.1
262454_at	At1g11190	bifunctional nuclease (BFN1)	1171.6	568.8	2.1

Prob set	AGI code	Gene description	microarray results		
			0.3 μM B	100 μM B	fold change
257206_at	At3g16530	legume lectin family protein	162.6	79.1	2.1
265213_at	At1g05020	epsin N-terminal homology (ENTH) domain	88	42.9	2.1
246110_at	At5g20140	SOUL heme-binding family protein	101.7	49.6	2.1
266720_s_at	At2g46790	pseudo-response regulator 9 (APRR9) / timing of CAB expression 1-like protein (TL1)	72.7	35.5	2.0
266218_s_at	At2g28850	cytochrome P450 family protein	133.6	65.7	2.0
263042_at	At1g23340	expressed protein	237	116.7	2.0
246708_at	At5g28150	expressed protein	1079.6	534.8	2.0
264263_at	At1g09155	SKP1 interacting partner 3	194.8	96.5	2.0
263963_at	At2g36080	DNA-binding protein	299.3	148.5	2.0
245550_at	At4g15330	cytochrome P450 family protein	417.1	207	2.0
263709_at	At1g09310	expressed protein	29.6	14.7	2.0
262717_s_at	At1g16410	cytochrome P450 family protein	536.4	266.7	2.0
249867_at	At5g23020	2-isopropylmalate synthase 2 (IMS2)	7052	3519.3	2.0

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