Hüseyin TOMBULOĞLU

ROLE OF MOBILE RNAS AND MYB TRANSCRIPTION FACTORS IN LONG-DISTANCE COMMUNICATION OF STRESS-INDUCED PLANTS

Ph.D.

FATİH UNIVERSITY

The Graduate School of Sciences and Engineering

Doctor of Philosophy in Biotechnology

ROLE OF MOBILE RNAS AND MYB TRANSCRIPTION FACTORS IN LONG-DISTANCE COMMUNICATION OF STRESS-INDUCED PLANTS

by

Hüseyin TOMBULOĞLU

January 2014

ROLE OF MOBILE RNAS AND MYB TRANSCRIPTION FACTORS IN LONG-DISTANCE COMMUNICATION OF STRESS-INDUCED PLANTS

by

Hüseyin TOMBULOĞLU

A thesis submitted to

The Graduate School of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Biotechnology

January 2014
Istanbul, Turkey

APPROVAL PAGE

This is to certify that I have read this thesis written by Hüseyin TOMBULOĞLU and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy in Biotechnology.

_____
Assoc. Prof. M. Serdal SAKÇALI
Thesis Supervisor

_____
Assoc. Prof. Turgay ÜNVER
Co-Supervisor

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy in Biotechnology.

_____
Assoc. Prof. M. Fatih ABASIYANIK
Head of Department

Examining Committee Members

Assoc. Prof. Ali TÜRKAN _____

Assoc. Prof. M. Serdal SAKÇALI _____

Assoc. Prof. Lokman ALPSOY _____

Assoc. Prof. İ. İlker ÖZYİĞİT _____

Assoc. Prof. Bayram YÜKSEL _____

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate School of Sciences and Engineering.

_____
Assoc. Prof. Nurullah ARSLAN
Director

January 2014

ROLE OF MOBILE RNAS AND MYB TRANSCRIPTION FACTORS IN LONG-DISTANCE COMMUNICATION OF STRESS-INDUCED PLANTS

Hüseyin TOMBULOĞLU

Ph.D. Thesis – Biotechnology
January 2014

Thesis Supervisor: Assoc. Prof. M. Serdal SAKÇALI

Co-Supervisor: Assoc. Prof. Turgay ÜNVER

ABSTRACT

Cell to cell communication is a strategy for plants which helps the regulation of the bioprocesses during plant development and adaptation to various environmental conditions. In this strategy, some molecules (RNAs and proteins) can move from one cell to another by using specific symplasmic network connected via plasmodesmata and alters the gene expressions. Other than the cellular communication, distance cells and even organs can communicate to each other via plant vascular bundle, phloem and xylem. In this mechanism, some RNAs, including viral RNAs, cellular mRNAs, tRNAs, microRNAs (miRNAs), small RNAs (smRNAs) and some transcription factors (TF) can be used as a signal molecule which moves over long distances. In this thesis, we aimed to analyze organ to organ communication strategy of plants under Boron (B)-stressed condition. For this aim, phloem-derived miRNA molecules were detected in *Cucurbita maxima* plants and they were quantitatively analyzed by RT-qPCR. Their target proteins were predicted by using bioinformatics tools and some miRNA molecules were found to be selectively expressed as a result to B-induction. In addition to these analyses, as a candidate signaling factor and/or regulator, R2R3-type MYB transcription factors (TF) were searched in the transcriptome data of B-tolerant barley cultivar, (*Hordeum vulgare*, cv Sahara). By using several bioinformatics tools, 51 R2R3 MYB TFs were identified and their possible roles against B stress were predicted. Expression analysis of those TFs was revealed that some MYB TFs were activated against B-induction. As a conclusion, in this thesis we hypothesized that some signaling molecules (miRNA and TFs) may be involved in the regulation of B-homeostasis under stress conditions. Some miRNA molecules and R2R3-type MYB TFs were found to be critical for B-stress

regulation and signaling. We hope that, these results can contribute to better understanding of signal transduction in long distance communications of plants.

Keywords: Long Distance Communication, MYB Transcription Factors, miRNA, Boron stress, phloem.

BİTKİLERDE UZAK MESAFE İLETİŞİMİNDE HAREKETLİ RNA' LARIN VE MYB TRANSKRİPSİYON FAKTÖRLERİNİN ROLÜ

Hüseyin TOMBULOĞLU

Doktora Tezi – Biyoteknoloji
Ocak 2014

Tez Danışmanı: Prof. Dr. M. Serdal SAKÇALI

Tez eş danışmanı: Prof. Dr. Turgay ÜNVER

ÖZ

Hücre-hücre iletişimi/etkileşimi, bitkilerde çevresel değişimlere karşı bitkilerin gelişimi ve adaptasyonu için gereklidir. Hücrelerarası etkin bir iletişim stratejisi olarak bitkiler plasmodesmata aracılığı ile birbirlerine bağlı özelleşmiş hücre içi iletişim mekanizmasına sahiptir. Bu yol ile hücreler arasında sınırlı bir bilgi alışverişi sağlanmaktadır. Uzak mesafeler arası (organlar arası) hızlı bir bilgi paylaşımı gerektiğinde ise ksilem ve floem aracılığı ile bu bilgi paylaşımı gerçekleştirilir. Bu zamana kadar, çeşitli bitki türlerinin floem sıvılarında bir dizi farklı RNA moleküllerine örneğin viral RNA' lar, hücresel kaynaklı mRNA' lar, mikroRNA' lar (miRNA), bir kısım küçük RNA' lar ve bazı transkripsiyon faktörlerine rastlanılmıştır. Bu tez kapsamında, bitkilerde Bor (B) stresi varlığında organlar arasındaki iletişim stratejisinin incelenmesi amaçlanmıştır. Bu amaçla, kabak bitkisi (*Cucurbita maxima*) floem sıvısında var olan miRNA' lar tespit edilmiş ve B stresindeki anlatım düzeyleri nicel olarak RT-qPCR yöntemine göre analiz edilmiştir. Tespit edilen miRNA' ların olası hedef genleri biyoenformatik araçlarla belirlenmiş ve bazı miRNA' ların B stresi varlığında ifade artışı gösterdikleri tespit edilmiştir. Bu analizlerin yanında, olası sinyal molekülü ve aynı zamanda düzenleyici R2R3-tip MYB transkripsiyon faktörleri B-dirençli arpa bitkisi (*Hordeum vulgare*, cv Sahara) transkripsiyon verileri içerisinde taranmıştır. Birçok biyoenformatik araç kullanılarak 51 farklı R2R3-tip MYB transkripsiyon faktörü tanımlanmış ve B stresindeki rolleri tartışılmıştır. Bu genlere ait ifade düzeyleri karşılaştırıldığında bazı R2R3-tip MYB transkripsiyon faktörlerinin B stresine karşı harekete geçtikleri belirlenmiştir. Sonuç olarak bu tez kapsamında bazı miRNA' ların ve TF' lerin bitkilerde stres koşullarında B-düzenlenme mekanizmasında, özellikle uzak organ iletişiminde rol oynayabileceği varsayılmıştır. Elde edilen bulgulara göre, bazı miRNA' ların ve R2R3-tip MYB transkripsiyon faktörlerinin B-

stresi düzenlenmesinde ve sinyalizasyonunda önemli oldukları bulunmuştur. Elde edilen sonuçların, bitkilerde uzak organ iletişimi ve sinyal iletimi mekanizmalarının daha iyi anlaşılabilmesine katkılar sunacağını beklenmektedir.

Anahtar Kelimeler: Uzak Organ İletişimi, MYB Transkripsiyon Faktörler, miRNA, Bor Stresi, Floem

To my parents and my dear wife

ACKNOWLEDGEMENT

I express sincere appreciation to Assoc. Prof. Dr. M. Serdal SAKÇALI and Assoc. Prof. Dr. Turgay ÜNVER for their guidance and insight throughout this dissertation.

I express my thanks and appreciation to my family and my dear wife for their understanding, motivation and patience. Lastly, but in no sense the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION	
AMV	Avian myeloblastosis virus
B	Boron
cDNA	Complementary DNA
DBD	DNA binding domains
dNTP	Deoxy-nucleotide tri phosphate
EDTA	Ethylenediaminetetraacetic acid
IEF	Iso-electric Focusing
miRNA	Micro RNA
mRNA	Messenger RNA
NAC	NAM, ATAF1/2, and CUC2
PlantTFDB	Plant transcription factor database
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RTase	Revers transcriptase
RT-qPCR	Revers transcriptase quantitative PCR
smRNA	Small RNA
TCA	Trichloroacetic acid
TF	Transcription Factor

CHAPTER 1

LITERATURE SURVEY

1.1 LONG DISTANCE COMMUNICATION IN PLANTS

Cell to cell communication is a strategy for plants which helps regulating the bioprocesses during plant development and adaptation to various environmental conditions. In this strategy, some molecules (RNAs and proteins) can move from one cell to another by using a specific symplasmic network connected via plasmodesmata and alters the gene expressions (Kehr and Buhtz 2008). Other than the cellular communication, distance cells and even organs can communicate to each other via plant vascular bundle, phloem and xylem. In this mechanism, some RNAs, including viral RNAs, cellular mRNAs, tRNAs, microRNAs (miRNAs), small RNAs (smRNAs) and some transcription factors (TF) can be used as a signal molecule which moves over long distances (Kragler, 2010; Kehr and Buhtz, 2008; Kehr, 2012). Up to know, the occurrence of these RNAs has been established in different plant species such as cucurbits, white lupin, castor bean, yucca (Yoo et al., 2004), oilseed rape (Buhtz et al., 2008), and recently apple (Varkonyi-Gasic et al., 2010; Kehr, 2012). For instance, heterograft experiment between pumpkin and cucumber plants demonstrated that a *CmNACP* transcript is found in scion and apical tissues of cucumber (Ruiz-Medrano et al., 1999). Also, it was shown that *KNOTTED1*-like homeobox transcripts can move in the scions of tomato and change the phenotype (Kim et al., 2001). In addition, overexpression of a patato *BEL1*-like transcription factor, move from leaf to root, increased the tuber formation (Banerjee et al., 2006).

In addition to mRNAs, in recent years some miRNAs emerged as a new type of signaling molecules that they can move over long distances. Buhtz et al., (2010) suggested that miR399 is a phloem-mobile long distance molecule involved in the

phosphate starvation response. In addition, several researchers showed the movement of small mobile RNAs in the signalization system of plants via phloem (Voinnet and Baulcombe, 1997; Palauqui et al., 1997; Yoo et al., 2004; Molnar et al., 2010). List of endogenous mRNAs and miRNAs found in phloem samples is indicated in Table 1.1.

Table 1.1 List of endogenous mRNAs and miRNAs found in phloem samples (Adapted from Kehr and Buhtz, 2008).

RNA	Function	Plant species	Reference(s)
Actin	Cytoskeleton	*O. sativa*	Sasaki et al., 1998
Aquaporin	Water transport	*H. vulgare*	Doering-Saad et al., 2002
BEL-1	Tuber development	*S. tuberosum*	Banerjee et al., 2006
CmCYCLINP	Cell cycle	*C. maxima*	Ruiz-Medrano et al., 1999
CmGAIP	Leaf development	*C. maxima*	Haywood et al., 2005
CmNACP	Meristem maintenance	*C. maxima*	Ruiz-Medrano et al., 1999
CmPDHPP	Regulation of glycolysis	*C. maxima*	Ruiz-Medrano et al., 1999
CmPP16	RNA transport	*C. maxima*	Xoxonostle-Cazares et al., 1999
CmRABP	Intracellular vesicular trafficking	*C. maxima*	Ruiz-Medrano et al., 1999
CmRINGP	Transcriptional regulation	*C. maxima*	Ruiz-Medrano et al., 1999
CmSTMP	Meristem cell fate	*C. maxima*	Ruiz-Medrano et al., 1999
CmSUTP1	Sucrose transport	*C. maxima*	Ruiz-Medrano et al., 1999
CmWRKYP	Defence response	*C. maxima*	Ruiz-Medrano et al., 1999
DELLA-GAI	Leaf development	*A. thaliana*	Haywood et al., 2005
H+ ATPase PPA1	Energy transformation	*H. vulgare*	Doering-Saad et al., 2002
miR156	Transcriptional regulation	*C. maxima*	Yoo et al., 2004
miR159	Transcriptional regulation	*C. maxima*	Yoo et al., 2004
miR167	Transcriptional regulation	*C. maxima*	Yoo et al., 2004
miR171	Transcriptional regulation	*C. maxima*	Yoo et al., 2004
OryzacystatinI	Protease inhibition	*O. sativa*	Sasaki et al., 1998
PFP-LeT6	Leaf development	*L. esculentum*	Kim et al., 2001
SUT1	Sucrose transport	*H. vulgare, S. tuberosum*	Doering-Saad et al., 2002; Kühn et al., 1997
Thioredoxin h	Redox regulation	*O.sativa, B.napus*	Sasaki et al., 1998; Giavalisco et al., 2006

1.2 SIGNALING MOLECULES OVER LONG-DISTANCES

Plant organs such as roots and leaves have to communicate with each other to sustain their life. This is achieved by the long-distance transport of signals, such as plant hormones, mRNAs, and miRNAs through the sieve tubes (Sjolund, 1997; Oparka and Turgeon, 1999; Suárez-López, 2005; Harada, 2010). Ruiz-Medrano et al. (1999) identified several mRNA molecules in the phloem sap through the characterization of the cDNA clones obtained from pumpkin (*Cucurbita maxima* Duchesne) sap. Yoo et al. (2004) demonstrated the presence of an endogenous population of small RNA species, ranging from 18 to 25 nucleotides in size, in the phloem sap of pumpkin, cucumber (*Cucumis sativus* L.), white lupin (*Lupinus albus* L.), castor bean (*Ricinus communis* L.), and yucca (*Yucca filamentosa* L.) (Kudo and Harada, 2007). In this section, mobile and long-distance transmissible molecules are introduced based on literature survey.

1.2.1 Long Distance Transport of mRNA

RNAs in the phloem sap have been discovered since 1960 and 70s (Ziegler and Kluge 1962; Kollmann et al. 1970). However, the techniques used in such studies attributed that the presence of RNAs in the phloem sap could be a contamination during the phloem sap collection. Because, it was an unexpected situation for plant science (Kehr, 2012). Afterwards, RNA presence in the phloem sap of plant has been demonstrated by several researchers. In such studies, RNA movement was proven by grafting experiments which is a technique used to fuse two different parts of the plants: the bottom part is called the rootstock which contributes roots and support, and the upper part is called the scion contributing stems, leaves, flowers, and fruits, (Harada, 2010). Transfer of mRNAs from rootstock to scion or *vice versa* have been shown in different plant species and for several mRNAs. Long-distance transportability of mRNAs proven using the grafting experiments were reviewed and listed by Harada (2010) (Table 1.2).

Table 1.2 Endogenous RNAs having long-distance transportability through sieve element, as demonstrated by experiments based on grafting (Harada, 2010).

Gene	Functional description	Plant used	References
	mRNA		
PP16	RNA movement protein showing paralog to viral movement protein	Pumpkin	Xoconostle-Cázares et al., 1999
NACP	Homolog of NAC domain protein	Pumpkin	Ruiz-Medrano et al., 1999
PFP-T6	Pyrophosphate-dependent phosphofructokinase (PFP)-LeT6 (KOTTED-1-like homeobox) fused gene	Tomato Potato	Kim et al., 2001 Kudo and Harada, 2007
GAI	Transcritional regulator of gibberellic acid response genes	Tomato, *Arabidopsis*	Haywood et al., 2005 Ham et al., 2009 Huang and Yu, 2009
BEL5	Transcription factor belonging to TALE family	Potato	Banerjee et al., 2006 Banerjee et al., 2009
AUX/IAA14, IAA18	Transcriptional repressor of auxin responsive genes	Melon Apple	Omid et al., 2007 Kanehira et al., 2010 Hannapel et al., 2013
	MiRNA		
miR399	Cleavage PHO2 mRNA to control inorganic phosphate homeostasis	*Arabidopsis* Tobacco	Lin et al., 2008 Pant et al., 2008
	SiRNA		
PAI	Phosphoribosylanthranilate isomerase tryptophan biosynthetic gene	*Arabidopsis*	Molnar et al., 2010
IR71	An inverted repeat on chromosome 3	*Arabidopsis*	Dunoyer et al., 2010

Heterografting experiments between cucumber and pumpkin demonstrated that *CmPP16* mRNA is transported as a signal molecule (Xoconostle-Cázares et al. 1999). In the same year, mRNA coding a *CmNACP*, homolog of NAC (NAM, ATAF1/2, and CUC2) domain protein has been shown to move over long distances (Ruiz-Medrano et al., 1999). Another heterografting experiment was designed using potato (*Solanum tuberosum)* as scion and tomato (*Lycopersicon esculentum)* as stock to test whether an RNA molecule responsible for changing leaf shape can be transported and function across the grafting junction. The results demonstrated that *PFP-T6* transcripts moved from the stock to the scion (Kudo and Harada, 2007). The same transcript has been reported by Kim et al (2001) to be involved in the changing of leaf morphology. Other than those mRNAs, *GAI* (*GIBBERELLIC ACID-INSENSITIVE*) transcript, transcriptional regulator of gibberellic acid response gene, has been firstly reported to be mobile along sieve cells in the stem of pumpkin (Ruiz-Medrano et al. 1999). Later,

detailed studies were indicated that *GAI* mRNAs are involved in pumpkin, tomato, and *Arabidopsis* (Haywood et al. 2005). Finally, the mobility of *GAI* transcripts was also observed in apples (Xu et al. 2010). However, unlike in the studies of *Arabidopsis*, pumpkin, and tomato, which showed directional *GAI* transport from stock to the scion, *GAI* RNAs were mobile through both directions (Lee and Zhou, 2012). In addition, *Aux/IAA* transcripts (*MpSLR/IAA14*, *IAA18*) were found to be transported into the root system where they suppress lateral root formation (Omid et al., 2007; Kanehira et al., 2010; Hannapel et al., 2013). Hannapel et al (2013) proposed that phloem transport of both *StBEL5* and *Aux/IAA* RNAs are linked to hormone metabolism and both targets the auxin synthesis gene or auxin signaling process (Hannapel et al., 2013).

1.2.2 Long Distance signaling via miRNAs

Expression of some miRNAs is induced by environmental stresses such as hypoxia (Moldovan et al., 2010), drought (Zhao et al., 2007; Kantar et al., 2010; Zhou et al., 2010), nutrient deficiency (Chiou, 2007) and salinity (Borsani et al., 2005; Jagadeeswaran et al., 2009; Zhao et al., 2009). In the case of phosphate and sulphate deficiency, it was demonstrated that some of those miRNAs are capable of moving long distances via the phloem (Buhtz et al., 2008; Buhtz et al., 2010; Gursanscky and Carroll 2012).

1.2.3 Long Distance signaling via Transcription Factors

One of the important long-distance signaling molecules in plants is transcription factors (TFs). Recent studies demonstrated that some TFs are involved in the phloem sap and they can move across the roots to leaf or *vice versa*. In potato (*S. tuberosum*), *StBEL5* transcripts, a *BEL1*-like homeobox gene which encodes a transcription factor, were found to be transported over leaves and petioles and stimulates the tuber formation at the stolon tips (Banerjee et al. 2006). This movement of RNA originates in leaf veins and petioles and is induced by a short day photoperiod, regulated by the untranslated regions, and correlated with enhanced tuber production (Banerjee et al. 2009). A grafting experiment fusing potato scion, over-expressing the *StBEL5* gene, with the wild type potato stock revealed that *StBEL5* transcripts are delivered to the target organ via the phloem stream in a long-distance signaling pathway (Banerjee et al. 2006).

1.2.4 Role of RNPs in Communication across the Plant Organs

To now, limited studies were conducted to identify the interaction of ribonuclear proteins (RNPs) and mRNAs during long distance signaling mechanism. Ham et al. (2009) reported that the polypyrimidine tract binding motif within the *GAI* mRNA is involved in the formation of a mobile ribonucleoprotein complex (Harada, 2010). Huang and Yu (2009) proposed the presence of motifs that are necessary and sufficient for long-distance trafficking of the *GAI* transcript (Harada, 2010). Gel mobility-shift assays have demonstrated the role of specific polypyrimidine tract-binding motifs in the pumpkin phloem-derived RNAs transcripts, *CmGAIP* and *CmPP16-1*, that the motif mediates binding of RNAs with RNPs (Ham et al., 2009). In this system, phloem RNAs and the CmRBP50 protein provide the basis of a mobile ribonucleoprotein complex containing as many as 16 proteins (Banerjee et al. 2009). Although not much is known about the protein partners of miRNAs, probably some protein/RNA complexes escort them through the vascular system of plants (David Hannapel, personal communication, 2012).

1.2.5 mRNA Special Motifs for Target Prediction

In the upstream region of target genes TTGAC motifs are found in the core structure. For instance, three light-repression motifs have been identified in the *StBEL5* promoter, GGGCC, ATAAAACGT, and another involved in shade-avoidance responses (Steindler et al., 1999). The stimulation of *StBEL5* gene was achieved by light induction. *StBEL5* gene expression was observed in leaf veins and petioles and the transcripts move along the phloem to roots where short-day photoperiod facilitates movement to stolon tips. During the phloem travelling of mRNA, some phloem chaperons may escort to direct the transcript towards to the site-specific targets, like stolon tips or roots (Ham et al. 2009; Lin et al. 2013). In roots, mRNAs are transcribed and attaches to Knox protein targets. BEL5/Knox protein complex regulates the transcription of some genes by binding to the tandem TTGAC core motif in the promoter region of target genes. Hannapel et al (2013) proposed 12 target genes which are included at least one TTGAC motif structure at their upstream positions (*StGA20ox1*, *StGA2ox1*, *YUCCA1a*, *YUCCA1c*, *IPT*, *ARF8*, *StPIN1*, *StPIN2*, *StPIN4*, *LAX1*, *LAX4*, *AGL8*, and *StBEL5*) (Hannapel et al. 2013; Lin et al. 2013).

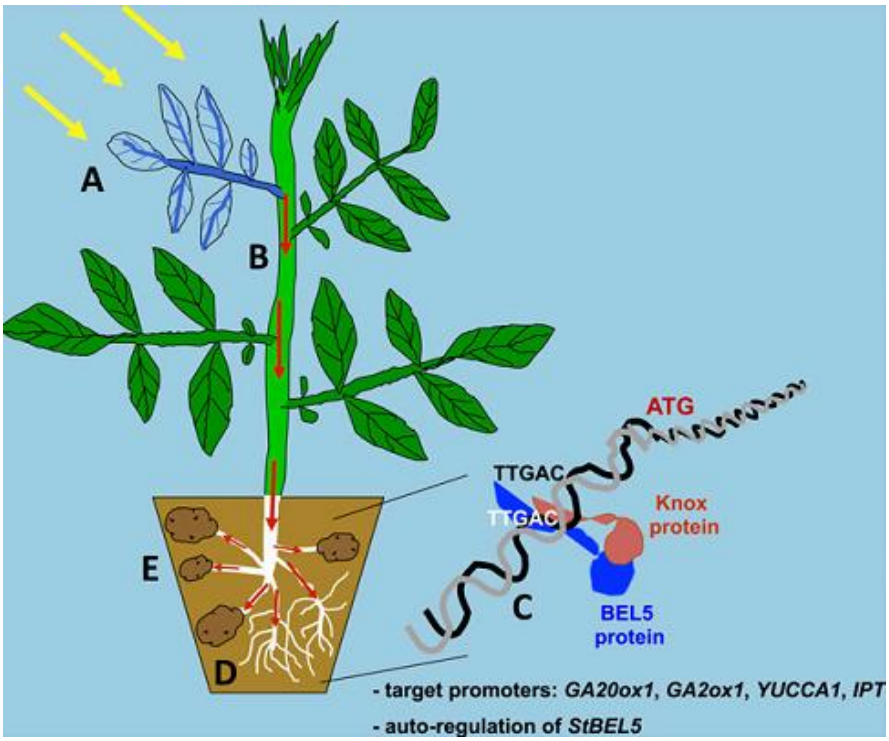

Figure 1.1 Schema summarizes the induction of root development and tuber formation thanks to the long distance trafficking of mobile *StBEL5* (Lin et al. 2013).

1.3 SMALL NON-CODING RNAs

Plant genomes encode several kinds of small RNAs ranging is sizes 21 to 24 nucleotides. They are classified into microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs) and repeat-associated siRNAs (rasiRNAs) (Vaucheret, 2006). Recently, Katiyar-Agarwal et al (2007) reported a novel class of small RNA, called as long-siRNAs (lsiRNAs) which are 30-40 nucleotides in sizes. Among small RNAs, miRNAs are well-studied one. They are found to be responsive in many biological processes in plants such as drought, salt, heat, ABA, hypoxia, cold, oxidative, UV-B radiation, nutrient-deprivation (sulfate, phosphate, copper, …etc.) stresses (reviewed by Sunkar, 2010) and biotic stresses such as bacterial pathogenesis (reviewed by Sunkar et al., 2012).

miRNAs target for the cleavage of its mRNA target and they re-regulates the mRNA translation at the post-transcriptional level. Induction or repression of miRNAs is critical for cellular responses and re-programming of bio-molecular processes against

changing conditions. As being a short nucleotide sequences, miRNAs are derived from single-stranded primary miRNA transcripts which are in a hairpin structure formed by the activity of a dicer –like (DCL) enzyme. DCL1, together with hyponastic leaves 1 (HYL1) and serrate (SE) proteins, accurately cuts out the miRNA-miRNA* duplex from the hairpin structure. HUA enhancer 1 (HEN1) adds methyl groups to the 3′ end of the duplex, which is exported to the cytoplasm. miRNA in the cytoplasm is then loaded into an RNA-induced silencing complex (RISC) containing Argonaute (AGO) protein (Shukla et al., 2008). The biogenesis of miRNAs has been reviewed by Jones-Rhoades et al (2006) and Chen (2005).

Stress-induced miRNA expression profiling in plants revealed that some miRNAs are induced only in specific organs and in special stress conditions (reviewed by Sunkar et al., 2012). Moreover, some miRNAs are synthesized in a tissue and move cell to cell or over long-distances. Cell to cell movement of miRNAs and siRNAs are maintained by plasmodesmata, the channels that connect the cytoplasm of adjacent cells, allow the passive diffusion of water, small solutes and the selective intercellular movement of macromolecules and viruses (Burch-Smith and Zambryski, 2012). Cell to cell movement of miRNAs has been firstly proposed by the studies of miR156/166 in maize and *Arabidopsis* (Kidner and Martienssen, 2004; Juarez et al., 2004).

1.3.1 Role of miR393 in Plant Stresses

In recent studies, miR393 was found to be associated with the pathogen resistance. In *Arabidopsis* leaves miR393* (complementary strand of miR393) accumulates at high levels and provide bacterial resistance to *P. syringae* (Navarro et al., 2006; Zhang et al., 2011). Its expression and accumulation was found to be transient. In general miR393 expressions were found to be upregulated in drought, salt, cold, heat, ABA and UV-B stresses (reviewed by Sunkar et al. 2012). miR393-mediated post transcriptional regulation was correlated with auxin perception and signaling. According the proposed mechanism, miR393 is upregulated in stresses and inhibits the auxin receptor *TIR1* (transport inhibitor response 1) which controls the auxin responsive genes via ARF transcription factor (ARFs) genes. Supression of *TIR1* causes the dimerization of *AUX/IAA* (auxin/indole-3-acetic acid) with *ARF* genes. ARF downregulation leads to repression of auxin signaling, and ARF mediated gene expression (Sunkar et al. 2012). Hence, the upregulation of miR393 in stresses effects

the ARF TF downregulation and causes to growth attenuation, auxin homeostasis and enhanced resistance to stresses (Sunkar et al. 2012) (Figure 1.2).

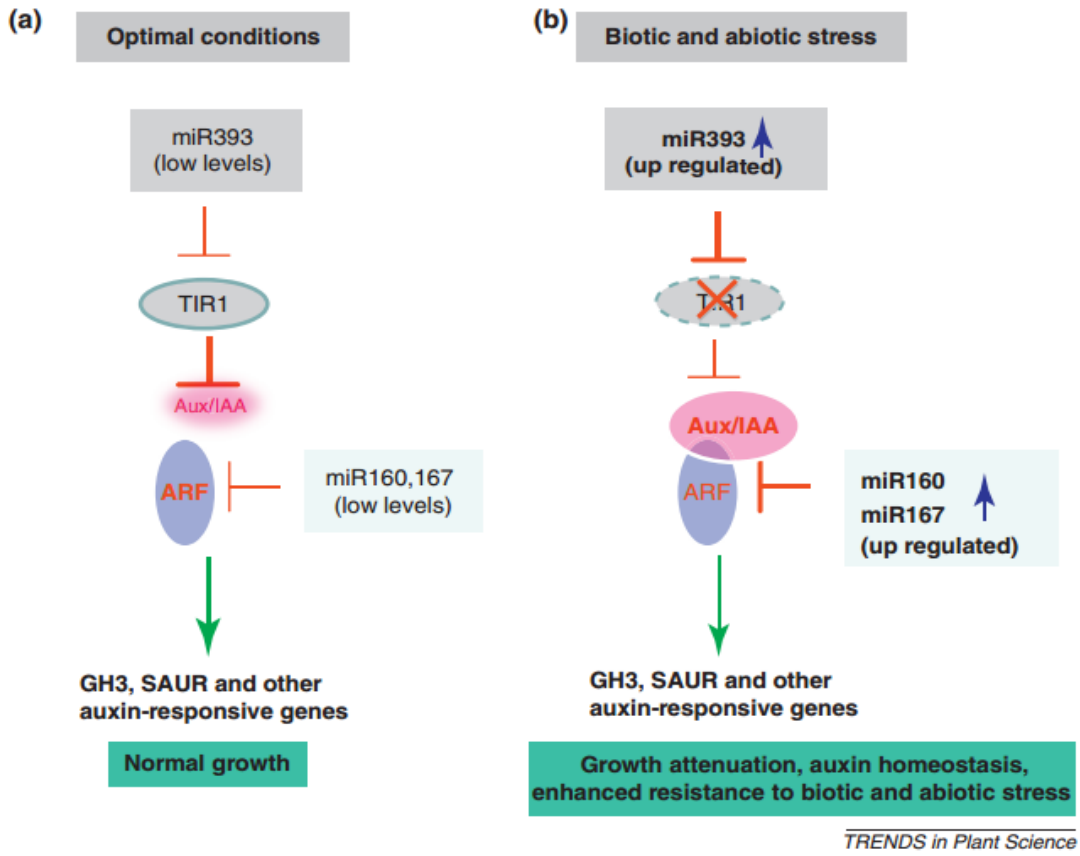

Figure 1.2 Proposed mechanism of miR393-mediated gene regulation. In optimal conditions, low level of miR393 mediates the auxin-responsive gene expression via ARF accumulation. In stressed conditions, miR393 level increases and causes to *TIR1* repression that lead to *Aux/IAA* heterodimerization. Low level of ARFs promotes to the enhanced resistance to biotic and abiotic stresses (retrieved from Sunkar et al. 2012).

In addition, miR393 expression was found to be increased with miR160 and miR167 upregulation in *Arabidopsis* infected by the PstDC3000 hrcC pathogen (Fahlgren et al. 2007). Also, treatment of *Arabidopsis* with flagellin-22 induced miR393 expression (Navarro et al. 2006). In the manner of plant-pathogen interaction, it seems that miR393 targets the auxin signaling gene repression which promotes to plant resistance against pathogen infection (Navarro et al. 2006; Ruiz-Ferrer and Voinnet, 2009).

1.3.2 Role of miR169 in Plant Stress

Recent studies showed that miR169 is a kind of regulatory miRNA, especially in stresses like drought and salt. miR169 response against drought induction is changing by plant to plant. For instance, drought stress caused upregulation of miR169 in *Oryza sativa*. However, the expression was found to be downregulated in *A. thaliana* (Li et al., 2008), *Medicago truncatula* (Trindade et al., 2010), and *Populus euphratica* (Li et al., 2011). Finally, miR169 level was found to be down-regulated only in roots (not in leaves) of drought stressed peace plant (*Prunus persica*) (Eldem et al., 2012). The conflict between the miR169 responses against drought may caused by the experiments carried out in different tissues. For instance in *Populus euphratica* the miRNA experiments were carried out using the leaf tissues (Li et al., 2011). Also, Eldem et al (2012) found that miR169 expression in leaves was not changed significantly in the leaves of drought-stressed peach. Although, there is a conflicting relation between the drought stress and miR169 expression, in common miR169 seems to be downregulated under drought stress, especially in roots rather than leaves. When miR169 is repressed, its target gene *NFYA5*, a transcription factor enhances the drought resistance in plants, is upregulated (Li et al., 2008). Over-expression studies of *NFYA5* revealed that the transgenic plants are drought-tolerant, whereas miR169-overexpression conferred the plant drought-sensitive (Li et al., 2008).

1.3.3 Mobile miRNAs

As well as to the cell to cell movement, mobile RNAs can move over long distances from root to shoot or *vice versa*. In recent years, some miRNAs were determined as mobile especially in deficient nutrient conditions. These studies have been focused on the conditions of phosphate, sulphate, copper and nitrate deprivation. It has been shown that phosphate deficiency caused miR399 accumulation in *Arabidopsis* (Bari et al., 2006). Pant et al (2008) simultaneously found that miR399 served as a long distance signal molecule in the phloem sap of rapeseed and pumpkin, for the regulation of plant phosphate homeostasis. In addition to the increased shoot phosphate levels, transgenic tomato overexpressing *Arabidopsis* miR399d has enhanced phosphorus accumulation by means of increased expression of P transporters and root proton exudation (Gao et al., 2010; Kehr, 2013). Another miRNA responded to P limitation in phloem sap is miR2111 (Pant et al., 2009). Similar to miR2111 and miR399, miR395

was found to be accumulated in vascular tissues, in this case upon sulfur deficiency (Aung et al., 2006; Buhtz et al., 2008; 2010). Grafting experiments revealed that miR395 is expressed in sulfur limitation and it can move in *Arabidopsis* vascular system unidirectional, from scions to rootstock (Buhtz et al., 2010).

1.4 BORON AS AN ENVIRONMENTAL STRESSOR

Boron (B), an essential micronutrient, is an environmental stress factor for plants. Toxicity and deficiency problems of B in agricultural lands have been reported in previous studies (Reid, 2007; Yau and Ryan, 2008; Cristobal et al., 2008). Excessive B can affect plant growth, development, productivity and causes yield loses for agricultural production (Sutton et al., 2007). One of the important crops that possibly suffer from B toxicity is barley (*Hordeum vulgare* L.). With its huge annual production all over the world (123,5 million tones) on an area of over 47,5 million hectares, among the cereals, it ranks fourth in the worldwide (FAOSTAT, 2012; http://faostat3.fao.org/). Barley genome, 5.1 Gb, is one of the largest in cereal crops and twice the size of the human genome (IBSC, 2012; www.barleygenome.org). Full genome sequencing has been undertaken by the International Barley Genome Sequencing Consortium (IBSC) and announced the date for completion by 2012 (Schulte et al., 2009; Schreiber et al., 2011). Finally, it was announced in October, 2012 and published (International Barley Genome Sequencing Consortium, 2012).

Up to know, three TFs have been identified related to B homeostasis in plants. AtWRKY6 TF involved in response to B-deficiency (Kasajima et al., 2010), NAC-like TF gene (Os04g0477300) functions when abolished by one-base insertion mutation, as B-toxicity tolerance in rice (Ochiai et al., 2011). The third is MYB-related genes, the expressions of AtMYB13 and AtMYB68 genes conferred boric acid tolerance on wild-type yeast (Nozawa et al., 2006). Apart from these data, as far as we know, no attempt has been made previously in the literature to show any other transcription factors that are involved in the regulation of B homeostasis in plants.

1.5 MYB TRANSCRIPTION FACTORS

Transcription factors (TF) are regulatory molecules of gene expression. They bind to either promoter or enhancer region of a gene and up/down regulates its expression. They have a modular structure (Latchman, 1997) and contain one or more DNA binding domains (DBD). By binding to DNA, they promote or repress the synthesis of mRNA, thereby regulating in almost all biological processes in cell. One of the largest transcription factor families is MYB. They found in all eukaryotes (Feller et al., 2011). MYB domain is the characteristic conserved DBD of MYB proteins. It is composed of 1-4 imperfect repeats of about 52 amino acids. Each of the repeats contains regularly spread triplet tryptophan (W) residues, forming a hydrophobic core structure (Kanei-Ishii et al., 1990). The tertiary structure of each repeats composed of three α-helices. The second and the third helices form the HTH (helix-turn-helix) structure and bind to its promoter target (Lipsick, 1996). The third helix is defined as a recognition helix that directly contact with DNA (Ogata et al., 1996; Williams and Grotewold, 1997; Jia et al., 2004; Dubos et al., 2010). In general, DBD spaced in the N-terminus of MYB proteins. The C-terminus is the flexible part of MYB transcription factor that function as trans-acting domain (TAD). Broad diversity of this domain among the MYBs gains the proteins wide distinct functions. Depending on the number of DBD repeats (R), MYB transcription factors have grouped into four classes (Dubos et al., 2010). The names of R1, R2, R3 come from c-Myb protein that is composed of three irregular MYB repeats. According to the similarity to the c-Myb repeats, MYB transcription factors called as 1R-MYB, R2R3-MYB, 3R-MYB and 4R-MYB. Among these, R2R3-MYB type transcription factors are the most common in plants (Du et al., 2012a).

The first gene described as containing a MYB domain was *v-myb* oncogene derived from avian myeloblastosis virus (AMV) (Klempnauer et al., 1982). In plants, the maize (*Zea mays*) C1 gene is the first plant MYB gene cloned, which is responsible for the regulation of anthocyanin pigmentation (Paz-Ares et al., 1987). Currently, in the plant transcription factor database (http://planttfdb.cbi.edu.cn/), 3485 MYB and 2754 MYB-related sequences are available (Zhang et al., 2011). They have many diverse functions in gene regulation processes including the control of plant metabolisms, environmental stress responses, hormonal responses and shaping cells and organs. The

extensive functions of MYB genes were reviewed by Du et al. (2009), Dubos et al. (2010) and Dai et al. (2012).

Recent studies have shown that MYB family transcription factors play roles in various environmental stresses such as: *AtMYC2* and *AtMYB2* are involved in ABA-regulated gene expression against drought and salt stresses (Abe et al., 2003); *AtMYB102* integrates signals derived from both wounding and osmotic stress (Denekamp and Smeekens, 2003); *AtMYB44*, *AtMYB60*, and *AtMYB61* are involved in the regulation of stomatal opening and closure (Cominelli et al., 2005; Liang et al., 2005; Jung et al., 2008); *AtMYB15* have role in cold stress and improves drought and salt tolerance in *Arabidopsis* (Agarwal et al., 2006; Ding et al., 2009); *AtMYB41* is involved in negative regulation of short-term transcriptional responses to osmotic stress (Lippold et al., 2009); *OsMYB2* encodes a stress-responsive MYB transcription factor having a regulatory role in tolerance of rice to salt, cold, and dehydration stresses (Yang et al., 2012); *OsMYBS3* confers cold tolerance in rice (Su et al., 2010); *OsMYB4* increases chilling and freezing tolerance in *Arabidopsis* and improves adaptive responses to drought and cold stress in apple (Vannini et al., 2004; Pasquali et al., 2008); *OsMYB3R-2* increases tolerance to freezing, drought, and salt stress in *Arabidopsis* (Dai et al., 2007); *StMYB1R-1* functions as a transcription factor involved in the activation of drought-related genes in potato (Shin et al., 2011). Constitutively expressed *AmMYB1* transcription factor provides better tolerance to NaCl stress in transgenic tobacco (Ganesan et al., 2012).

In this study, we focused on the whole transcriptome-wide identification of R2R3-type MYB TFs in barley. Additionally, we interested in the interaction of MYB TFs with B toxicity response. Sequences of the R2R3-type MYB transcription factors were isolated from the full RNA-seq data of barley, both non-stressed (control) and toxic concentration (1000 µM) treated root/leaf samples. As a result, 51 R2R3-type MYB transcription factors were identified from barley full transcriptome via protein motif and conserved domain searches. Phylogenetic analysis was conducted by combining the barley MYB sequences with *Arabidopsis*. The identified 51 barley R2R3 MYB TFs were divided into 34 subgroups. Conserved domains of the MYB TFs were compared with the DNA binding domain (DBD) of *Arabidopsis thaliana, Brachypodium distachyon* and *Zea mays*. We observed that *Arabidopsis*, *Brachypodium* and maize

DBDs showed high conservation with barley. Chromosome distribution and possible location of the *Myb* genes were predicted with comparing their *Brachypodium* homologs. In addition to expression data obtained from the deep sequencing, some of randomly selected barley MYB TFs was quantitatively measured and transcript accumulations were compared between the non-stressed (control) and B-induced root and leaf samples, separately. Since the MYB TFs are involved in diverse biological processes in plants, our findings will help the understanding of functionality of MYBs in plants against not just to B stress and also to many different biological regulation mechanisms.

Small changes of B cause many disorders and yield losses in all plants, especially in sensitive cereals. Although, many B-related genes and proteins have been identified, molecular RNA signalization mechanism for B regulation is not known yet. In this study, we aimed to identify mobile RNAs and MYB TFs involved in Boron homeostasis in plants. miRNA analysis of phloem exudates were conducted to find possible B-related mobile RNAs in the phloem sap of pumpkin (*Cucurbita maxima*). In addition, transcription factors may have role to the control of B regulation. One of a candidate transcription factor involved in phloem sap is MYB transcription factor. To profile the MYB transcription factors, several bioinformatics tool were used to identify MYB transcription factors in barley. Analyses were conducted on tolerant-barley cultivar (*H. vulgare* L. Sahara) under sufficient and high B conditions in hydroponic conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1 TRANSCRIPTOME-WIDE IDENTIFICATION of R2R3-MYB TFs IN BARLEY

2.1.1 Identification of MYB TFs in barley

Barley (*Hordeum vulgare* L. Sahara landrace) was kindly provided from Dr. Tim Sutton of Adelaide University-Australia. The seeds were planted in soil and then the seedlings were transferred into hydroponics supporting conditions. Two weeks later, toxics level of Boron (1 mM) was applied and plant organs (root and shoot) were harvested. Then total RNA isolations were carried out using Trizol® reagent (Invitrogen). Four sets of RNA libraries (50 µM root, 50 µM leaf, 1000 µM root, 1000 µM leaf) were constructed and sequenced by using Illumina Solexa® deep sequencing platform. MYB TF proteins were searched from annotated protein sequences of the full-transcriptome data. DNA-binding domain (DBD) of MYB was taken from the MYB consensus sequences referring to plant transcription factor database (PlantTFDB v2.0) (http://planttfdb.cbi.edu.cn/) (Zhang et al., 2011) (rgrWTteEdellvdavkqlGggtWktIartmgkgRtlkqcksrwqkyl - about 48 aminoacids). The conserved motif for MYBs was used as query and totally 320 MYB related protein sequences were filtered. Afterward, the selected MYB sequences were performed a BlastP search (BLASTP 2.2.27) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). 312 hits were collected that showed significant homology with DBD of barley MYBs. Only hits with e-values of <1.0 were considered to be members of MYB family and 276 non-redundant MYB sequences were obtained. Structural and functional

verification of the MYB domains were also predicted by using ScanProsite (release 20.83) (http://prosite.expasy.org/scanprosite/) (Apweiler et al., 2001) and SMART tools (http://smart.embl-heidelberg.de/) (Letunic et al., 2012).

2.1.2 Protein Motif Identification

For the conserved motif identification, MYB proteins were searched by using MEME tool (version 4.8.1) (http://meme.nbcr.net/meme/intro.html) (Bailey et al., 2006). Architecture of MYB proteins was determined with setting the parameters as adjusted: the distribution of motifs, 0 or 1 per sequence; minimum width of motif, 6; maximum width of motif, 250; maximum number of motifs to find, 50. Only motifs with an e-value <1e–10 were collected for further analysis. Subsequently, the MAST program (version 4.8.1) (http://meme.nbcr.net/meme/cgi-bin/mast.cgi) was used to align the conserved motifs of the proteins (Timothy and Gribskov, 1998). DBD of R2R3-type MYB TFs were compared with the results of barley. MYB protein sequences belonging to *Arabidopsis thaliana*, *Zea mays* and *Brachypodium distachyon* were taken from PlantTFDB (http://planttfdb.cbi.edu.cn/) (Zhang et al., 2011). Each of MYB group was aligned with ClustalW multiple sequence alignment tools. Only the DBD sequences were taken to display the consensus sequence analysis. Weblogo 3 program was used to compare the DBD of the species (Schneider and Stephens, 1990; Crooks et al., 2004).

2.1.3 Multiple Sequence Alignment and Phylogenetic Analysis of R2R3-type MYB TFs

Phylogenetic trees were produced individually by using DNA-binding domain and/or the full protein sequences of R2R3-type MYB TFs. 51 barley R2R3-MYB proteins were performed multiple alignment analysis with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). According to the data obtained with ClustalW, sequences containing partial MYB motifs were manually removed. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA Beta4 (version 5.1) (http://www.megasoftware.net) (Tamura et al., 2011). Neighbor-Joining (NJ) tree was constructed with the following parameters: Substitution, Poisson Model; Data subset to use, complete deletion; Replication, bootstrap analysis with 1000 replic

2.1.4 Expression Analysis of Barley MYB TFs

RPKM (Reads Per Kilobase per Million mapped reads) values were compared for each sample (50μM root; 50 μM leaf; 1000 μM root; 1000 μM leaf): B treated samples (1000 μM root; 1000 μM leaf) with control groups (50 μM root; 50 μM leaf) and samples within the each groups. To verify the transcriptome results, six MYB TFs were randomly selected and quantitatively analyzed by using RT-qPCR.

2.1.4.1 Primer Design

Primers for the six selected transcripts were designed considering with the non-conserved regions of MYB proteins. Primer design and RT-qPCR protocols were applied with minor changes according to our recent study (Tombuloglu et al., 2012). Partial sequences (approximately 120 bp) of six MYB transcripts were amplified by using reverse and forward primers. Primer sequences were designated by using a web-based program Primer3 (version 1.1.4.) (Rozen and Skaletsky, 2000). GC % and Tm values of primers were around 60 and between 58 and 60 °C, respectively. The sequence of target genes for barley was obtained from the full transcriptome data. The results were normalized by using *18S rRNA* barley gene (AY552749.1). Primer sequences used in this study were indicated in Table 2.1.

Table 2.1 Primers used in qRT PCR to validate transcriptome expression results (TM, melting point).

Primer	**Gene**	**Sequence 5'-3'**	**TM (°C)**	**Product (bp)**
CL32841-F	**HvMYB35**	GGCATTGTATGGCCAGCTC	58,96	102
CL32841-R		AGGTTCTCCGACACGTCCA	60,53	
CL7611-F	**HvMYB51**	CTAATGCTTCCACCACCCCA	59,67	103
CL7611-R		TTCCTTCACCAAGCTCCTCG	59,68	
CL25757-F	**HvMYB47**	ATGGAGCCCTGTTGAAGATG	57,57	85
CL25757-R		GCCGATCATTCTTCCGATTTC	57,47	
CL22262-F	**HvMYB45**	CCAATCTGGTGATGAGGGACG	60.47	194
CL22262-R		AGATGCCCATTTGTTCCCATGA	60.29	
CL4626-F	**HvMYB14**	GTCGCTGTCTGGGTACAACA	59.97	119
CL4626-R		TCGAGTTTAGCGTGCTGGAG	60,11	
CL20.15-F	**HvMYB09**	CACATGAGGAAGAAGGCCCA	59,67	180
CL20.15-R		GTCGCACATGCTAGTCTGC	59,00	
18S RNA-F	**18S-RNA**	GGCTACCACATCCAAGGAA	57,5	193
18S RNA-R		CTATTGGAGCTGGAATTACCG	58,0	

2.1.4.2 First strand cDNA synthesis

Before first strand cDNA synthesis, RNA integrity was verified on a 1% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. RNA concentration was calculated by using Cubit flour metric system (Invitrogen). 5 µg of total RNA was treated with five units of RNase-free recombinant DNase I (Roche) and incubated at 37 °C for 15 min. Reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0) to a final concentration of 8 mM and heating up to 75 °C for 10 min. The first strand of cDNA was synthesized using PrimeScript™ RT-PCR Kit (Takara Bio Inc.) according to suggestions of the manufacturers' protocol: 10 mM dNTP and 2.5 mM oligo dT primer with 1 µg of total RNA mixture in a 10 µl reaction volume was incubated at 65 °C for 5 min and added 5xPrimeScript™ Buffer, 20 units RNase inhibitor, 0.5 µl PrimeScript™ RTase and RNase-free water up to 20 µl for per sample. Reverse transcription was performed in a thermal cycler (Techne® TC512) using the following program: 42 °C for 30 min, 95 °C for 5 min and +4 °C as a final hold. Tubes were stored at -20 °C until further use.

2.1.4.3 Real-time reverse transcription-qPCR

Prior to quantification analysis, 7 MYB transcripts were amplified with a conventional PCR (Techne® TC512 Gradient Thermal Cycler), checked on 2% agarose gel. Afterwards, for quantification analysis, specific regions of targeted genes were amplified using SYBR® Premix Ex Taq™ kit (Takara Bio Inc.) and real-time PCR was performed in a Rotor-Gene® PCR machine (Corbett Research-Qiagen). Accordingly, 10 µl of SYBR® Premix Ex Taq™ (2×) (Tli RNaseH Plus) was mixed with 1 µl gene specific forward and reverse primers (10 µM each), and 2 µl of cDNA template. Sterile distilled water was added onto the mixture up to 20 µl. qPCR conditions consisted of a 95 °C for 10 min, 40 cycles of 95 °C for 7 s, 58–62 °C for 15 s, 72 °C for 10 s and a melting analysis of 52 to 95 °C with an increasing temperature 0.5 °C min^{-1}. Relative quantity of the individual transcripts was calculated by a mathematical model, which included an efficiency correction and crossing point (Cp) deviation of an unknown sample versus to a control (Pfaffl, 2001). Real-time efficiency score for individual transcripts was calculated by means of dilution series of cDNA templates (40; 4; 0.4; 0.04; 0.004 ng $µl^{-1}$). Slope of a standard curve of a dilution series was used to determine

the reaction efficiency (E) as a formula: $E=10^{(-1/slope)}$. Relative expression ratio of a target gene was computed according to the method stated by Pfaffl (2001).

2.1.5 Boron Treatment and Sample Collection

Seeds of B tolerant barley (*Hordeum vulgare* L. Sahara) were grown in soil for two weeks. Then the seedlings were transferred onto a hydroponics support containing macronutrient solution in mM: 6 KNO_3, 1 $NH_4H_2PO_4$, 2 $MgSO_4$, 4 $Ca(NO_3)_2$; and micronutrient solution in μM: 50 H_3BO_3, 9 $MnCl_2$, 0.3 $CuSO_4$, 25 Fe-EDTA, 0.8 $ZnSO_4$ and 0.02 g MoO_3 (85%). After two weeks for acclimatization, seedlings were exposed to 1000 μM boric acid (H_3BO_3) for 24 h. The pH of the nutrient solution was checked before and after boric acid addition and it was kept a constant pH at 5.8. Roots and leaves were harvested, directly treated with liquid nitrogen and then stored at −80 °C until RNA isolation.

2.1.6 RNA Extraction

Total RNA of root and leaf (treated with 50 μM and 1000 μM B concentration) samples were extracted with Trizol® (Invitrogen) reagent according to suggested procedures by manufacturer. Extracted RNA was dissolved in RNase-free water and stored at −80 °C. RNA integrity was verified on a 1% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. 1 μg of total RNA samples were treated with 1 μl of DNase I (Fermentas) and incubated at 37°C for 30 minutes. Reaction was stopped by adding 1 μl of 25 mM EDTA by heating at 65°C. All RNA samples were quantified, then examined for protein (A260/A280 nm ratios) and reagent contamination (A260/A230 nm ratios) by spectrophotometer.

2.1.7 Statistical Analysis

Statistical analysis was carried out using the data obtained from three separate cDNA sets of three independent biological samples. Nonparametric Kruskal–Wallis test was performed by using the SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL) program package. $P < 0.05$ was considered to be statistically significant.

2.2 MICRO-RNA (miRNA) DETECTION AND EXPRESSION ANALYSIS

2.2.1 Plant Sampling and RNA Isolation from Phloem Sap (PS) of *C. maxima*

Cucurbita maxima (DILL'S ATLANTIC GIANT) seeds were obtained from the Seed needs (USA). Prior to germination, surface sterilization was handled by washing with 70% ethanol and then with 5% bleaches (sodium hypochloride) for 15 minute and washed tree times with dH_2O to get rid of bleach remnants. Plants were sown into soil including pots and incubated under greenhouse conditions. Greenhouse (WiseCube, Lab Constant Temparature & Humudity Chamber, Korea) conditions were adjusted as %60 humidity; 23 ºC temperature; 16:8 (light:dark) photoperiod and 6000 Lux as intensity of light. Seedlings were watered three times a week periodically with a 1/3 Hoagland solution. The composition of Hoagland solution was adjusted with some modifications referred to Hoagland and Arnon (1950). For Hoagland medium: macronutrient and micronutrient stock solutions were prepared as follows: 6 mM KNO_3, 1 mM $NH_4H_2PO_4$, 2 mM $MgSO_4$, 4 mM $Ca(NO_3)_2$ for macronutrient solution; 15 µM H_3BO_3, 9 µM $MnCl_2$, 0,3 µM $CuSO_4$, 25 µM Fe-EDTA, 0.8 µM $ZnSO_4$, 0.02 g MoO_3 (85%) for micronutrient solution.

Figure 2.1 Two week old pumpkins before BA (Boric acid) application.

200 ml of 5 mM BA (Boric acid) was added to the soil and the phloem sap was collected even after 24 hours (Figure 2.1). Phloem sap was pooled from the three pumpkin plants with 30 min time intervals into a pre-cooled tube. The protocol for RNA isolation from phloem sap was carried out according to the method stated by Zhang et al 2009. The method was based on Trizol® LS Reagent (Invitrogen) phenol, guanidine isothiocyanate RNA isolation procedure developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The protocol was applied according the following procedure:

25 µl of fresh PS (phloem sap) with 75 µl of Trizol® LS reagent (Invitrogen) (1:3) mixed by vortex. The mixture was incubated for 5 min at room temperature. Then, 25 µl of chloroform (Merck, Germany) was added and the solution was shaken by hand for 15 seconds. The mixture was incubated for 15 min and then the sample was centrifuged at 12000xg for 15 min at 4 °C. The upper phase was collected into a new tube and 2x volumes of 99% ethanol (Millipore) were added. The sample was mixed by vortex and then it was incubated at -20 °C for overnight. Then the mixture was centrifuged at 16000xg for 30 min at 4 °C. The pellet was washed with 100 µl of lab-made %70 DEPC-treated ethanol (Millipore). The pellet with wash was centrifuged at 7500xg for 5 min at 4 °C and the supernatant was discarded. The pellet was air-dried for 10 min and it was re-suspended with 25 µl of nuclease-free water (Fermentas). To dissolve the RNA pellet, the solution was mixed by pipetting up and down for several times. Then the homogenate was thoroughly incubated at 60 °C in a water bath for 10 min. The RNA sample was stored in -80 °C until further applications.

2.2.2 RNA Quality and Concentration Calculation

Phloem-derived RNA and tissue specific RNAs (root, leaf) were pooled from three *C. maxima* plants. After RNA isolation, the concentration and quality of RNA were checked by NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific). The concentrations were noted as listed in Table 2.2

Table 2.2 RNA concentrations and absorbance values.

Sample	A_{260}	A_{280}	$A_{260/280}$	$A_{260/230}$	Concentration (ng/µl)
CucPS-C	3.02	1.6	1.88	0,28	146
CucPS-24h	0,49	0,32	1,51	0,15	24,2
CucR-C	4.23	2.14	1.97	1.84	177
CucR-24h	9,56	5,11	1,87	1,09	388
CucL-C	10,5	5.14	2.05	2.16	440
CucL-24h	18,6	9,2	2,01	1,54	745

2.2.3 RT-PCR Contamination Test

CmPP16 (Acc No: AF079170) and *RuBisCo* transcripts were used to analyze against any contamination during the phloem sap exudation and/or isolation. In here, *CmPP16* gene represented as a positive control while it is known as a phloem-specific gene observed in the phloem sap of Cucurbitacea species (Ruiz-Medrano et al 1999; Zhang et al 2009). In addition, *RuBisCo*, a green tissue-specific transcript, was selected as a negative control to test whether there is a contamination during the sampling process of phloem sap (for primer sequences, see in Table 2.3).

2.2.4 Stem-loop RT-qPCR Analysis

2.2.4.1 Stem-loop First Strand cDNA synthesis

Phloem-derived *C. maxima* RNAs were used to analyze the B-stress induced miRNAs. The method was developed by Varkonyi-Gasic et al. (2007) and it is most efficient method when starting from very small amount of sample RNA. In this experiment, 30 ng of total RNA for each sample (CucPS-C and CucPS-24h) were used to the synthesis of first strand cDNA. According to the method stated by the manufacturer (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific), 1,2 µl and 0,25 µl of RNAs were taken for the stem-loop RT PCR analysis of CucPS24h (B-stressed *C. maxima* phloem sap) and CucPS-C (Control *C. maxima* phloem sap), respectively. 1 µl (1 µM) of miRNA specific RT (reverse transcription) primers were added with 0,5 µl of dNTP mixture (10 mM) (Thermo Sci.) and the total volume was adjusted to 14 µl with DEPC-treated water. The mixture was first incubated at 65 °C for

5 min and immediately kept on ice for 2 min. It was then centrifuged to pick all solution down. Afterwards, 4 µl of 5x Reaction Buffer, 1 µl RNase inhibitor (RiboLock RNase Inhibitor (20 U/µl)), 1 µl of reverse transcriptase enzyme (RevertAid M-MuLV Reverse Transcriptase (200 U/µl)) were added respectively. 20 µl of total mixture was mixed thoroughly and spin down. The tubes were incubated at 16 °C for 30 min. Then the following reactions were applied to each sample tube.

30 °C for 30 sec
42 °C for 45 sec] 60x
48 °C for 1 sec

The reactions were processed in a thermal cycler (Techne) and the reaction was terminated at 70 °C by incubation the tubes for 5 min. The samples were stored at -20 °C until next processes.

2.2.4.2 Stem-loop qPCR Analysis

Quantitative accumulation of miRNAs upon B-stressed condition was assessed by performing the relative expression analysis of miRNA transcripts. For this aim, only 30 ng of RNA was used for each sample. The protocols for stem-loop RT-qPCR were applied with minor changes according to the method stated by recent publications (Varkonyi-Gasic et al 2007; Yanik et al 2013). Other than those methods, in here we used different enzymes supplied with different companies. Accordingly, Takara SYBR® Premix Ex Taq™ (2×) (Tli RNaseH Plus) kit were used to amplify miRNA molecules. The method was carried out by following the procedure: 10 µl of SYBR® Premix Ex Taq™ (2×) (Tli RNaseH Plus), 1 µl gene specific forward and universal reverse primers (10 µM each), and 2 µl of cDNA template. Sterile distilled water was added onto the mixture up to 20 µl. qPCR conditions consisted of a 95 °C for 2 min, 40 cycles of 95 °C for 5 s, 56 °C for 15 s, 72 °C for 20 s and a melting analysis of 52 to 95 °C with an increasing temperature 0.5 °C min^{-1}. The analysis were carried out using a Rotor-Gene® PCR machine (Corbett Research-Qiagen).

2.2.5 Target prediction of miRNAs

To find the possible miRNA targets, we searched the miRNA sequences by using psRNATarget (Plant Small RNA Target Analysis Server) server in the "User submitted small RNAs preloaded samples" platform (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011). The miRNA sequences were obtained from Jagadeeswaran et al (2012). The sequences were given in below:

>MIR156 UGACAGAAGAGAGUGAGCAC

>MIR159 UUUGGAUUGAAGGGAGCUCUA

>MIR160 UGCCUGGCUCCCUGUAUGCCA

>MIR162 UCGAUAAACCUCUGCAUCCAG

>MIR164 UGGAGAAGCAGGGCACGUGCU

>MIR166 UCGGACCAGGCUUCAUUCCCC

>MIR167 UGAAGCUGCCAGCAUGAUCUG

>MIR168 UCGCUUGGUGCAGGUCGGGAA

>MIR169 AAGCCAAGGAUGAAUUGCCGG

>MIR172 GGAAUCUUGAUGAUGCUGCAU

>MIR390 AAGCUCAGGAGGGAUAGCGCC

>MIR393 UCCAAAGGGAUCGCAUUGAUCC

>MIR397 UCAUUGAGUGCAGCGUUGAUG

>MIR398 UGUGUUCUCAGGUCACCCCUG

Table 2.3 Primers used for miRNA qPCR analysis.

miRNA	Forward P.	RT primer	Reference
miR156a	GCGGCGGTGACAGAAGAGAGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC	Varkonyi-Gasic 2010
miR159a	CGGCGGTTTGGATTGAAGGGA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC	
miR160a	TTCCTTGCCTGGCTCCCTGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGCAT	
miR162a	CGGCGTCGATAAACCTCTGC	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGAT	
miR164a	TGACGTTGGAGAAGCAGGGCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCACG	
miR166a	TCGCTTCGGACCAGGCTTCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA	
miR167a	TCGCGTGAAGCTGCCAGCAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGATC	
miR168a	GCGGCGGTCGCTTGGTGCAGGT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCG	
miR169a	GCGGCGGCAGCCAAGGATGACT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCA	
miR172a	CGGCGCAGAATCTTGATGATG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG	
miR390a	CGGCGAAGCTCAGGAGGGAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCGCT	
miR393a	GCGGCGGTCCAAAGGGATCGCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATCAA	
miR397a	CGGCGTCATTGAGTGCAGCG	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA	
miR398a	GCGGCGGTGTGTTCTCAGGTCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGGGG	
CmPP16 (positive control)	ATGGGTTTGAAGAAGCCAAGCCACTTA (Acc. AF079170)	GTGGTAAAGGACTTCAAGCCCACGACC (Acc .AF079170)	Ruiz-Medrano et al., 1999
RUBISCO (negative control)	TTGTCGAAGCCAATGACTCTGATGAA	ATGGCTTCCATCGTCTCATCCGCC	
Universal reverse		GTGCAGGGTCCGAGGT	

CHAPTER 3

RESULTS

3.1 TRANSCRIPTOME-WIDE MYB IDENTIFICATION IN BARLEY

3.1.1 Identification of R2R3-type MYB TFs in Barley

According to the data obtained from the full transcriptome profiling of barley (Kekeç et al. 2014, unpublished), consensus motif for MYB domain (around 48 amino acids) was searched as queries by using non-redundant protein sequence (nr) database of BlastP (2.2.27) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997). Among 148,285 contigs and 79,214 unigenes (1,311 distinct clusters and 77,843 distinct singletons), totally 320 MYB and MYB-related candidate protein sequences were filtered. Subsequently, all annotated MYB sequences were performed a second round of BlastP alignment with DNA-binding domain (DBD) of MYBs Only hits with e-values of < 1.0 were considered as members of this gene family and 276 non-redundant MYB sequences were obtained. Structural and functional verification of MYB domains were also predicted by using ScanProsite and SMART tools. While R2R3 and 3R MYB family TFs have apparent 2 or 3 consensus DBD repeats, sequences including single MYB domain or somewhat different conserved domains were discarded from the data list. Afterwards, we used the multiple sequence alignment tool (ClustalW) in order to validate the consensus MYB repeats (Figure 3.1). Sequences including gaps and ambiguously aligned sites were manually removed from the total barley MYB list. The sequences including the two DBD MYB domains were confirmed to be R2R3-MYB TF members. Finally, we demonstrated fifty one non-redundant R2R3 MYB transcription factors and they were named. The nomenclatures of barley MYBs were applied permanently according to their similarity analysis in ClustalW (Figure 3.1).

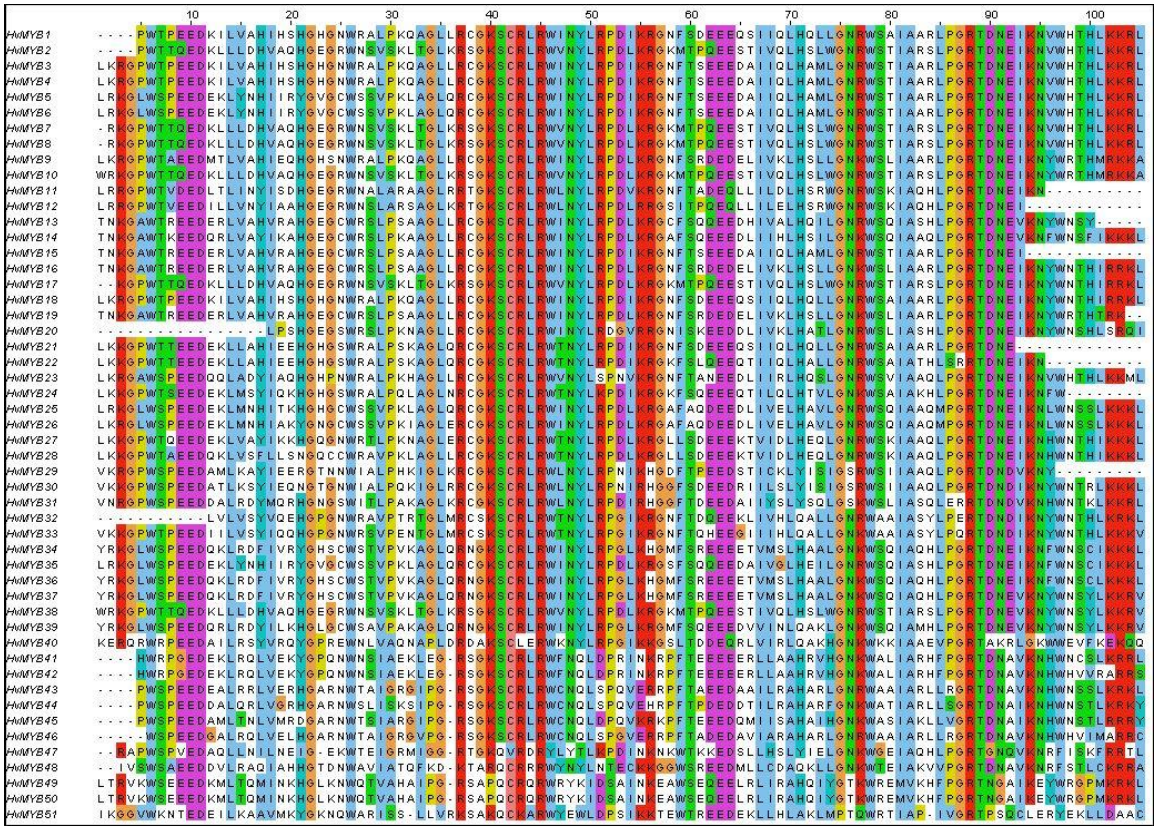

Figure 3. 1 Multiple alignment of 51 R2R3 MYB proteins.

3.1.2 DNA Binding Domain (DBD) and Conserved Residues in MYB Proteins

The main characteristics of R2R3-type MYB TFs include N-terminal positioned two DBD repeats which are around 105 amino acids in length. They include highly conserved triplet tryptophan (Trp, W) residues on each DBD repeat. In accordance with that features, MYB-specific characters were observed over all selected barley MYB sequences (Figure 3.2 A-H). The characteristic Trp (W) residues were positioned at 6, 26, and 46 for the R2 repeat and 59, 78 and 97 for the R3 repeat. Logo analysis was applied for DBD of four selected plants (*A. thaliana*, *B.distochyon*, maize and barley).

The results revealed that triplet Trp (W) residues were identical for R2 and R3 repeats. Beside, in the R2 repeat only seven residues were found to be changed among the selected plant MYB sequences (Figure 3.2 A-H). These changes generally occurred as substitutions between two or three residues. Through the third helix of R2 repeat of

the plants, only one residue was differed in barley. It was found that Ile47 (I) was preferred instead of Thr (T). The others were almost the same in terms of dominant residues. It revealed that the last part of R2 (from 34 to 53) was most conserved in the plant species. In the barley R2 sequence, except the six residue substitutions, any other unusual change was observed in comparison with the other plant MYBs (Figure 3.2 A, H). The upstream position of R2 repeat and complete part of R3 repeat was found to be highly conserved among the selected plants (*Arabidopsis*, *Brachypodium*, maize and barley). In the R3 repeat of all species, the first trytophan (Trp59) residue was not clear. However, the second and the third trytophan residues was apparent and showed high conservation. The total number of conserved residues of R3 repeat was much more than the R2 repeat. Also, dominant residue was not changed in the upstream part of R3 starting from 85 to 103. The region included one third of helix-2 and complete part of helix-3 were the most consistent part among the selected plants. Due to the functions of recognition and direct contact with DNA, helix-3 was also found to be highly conserved in barley.

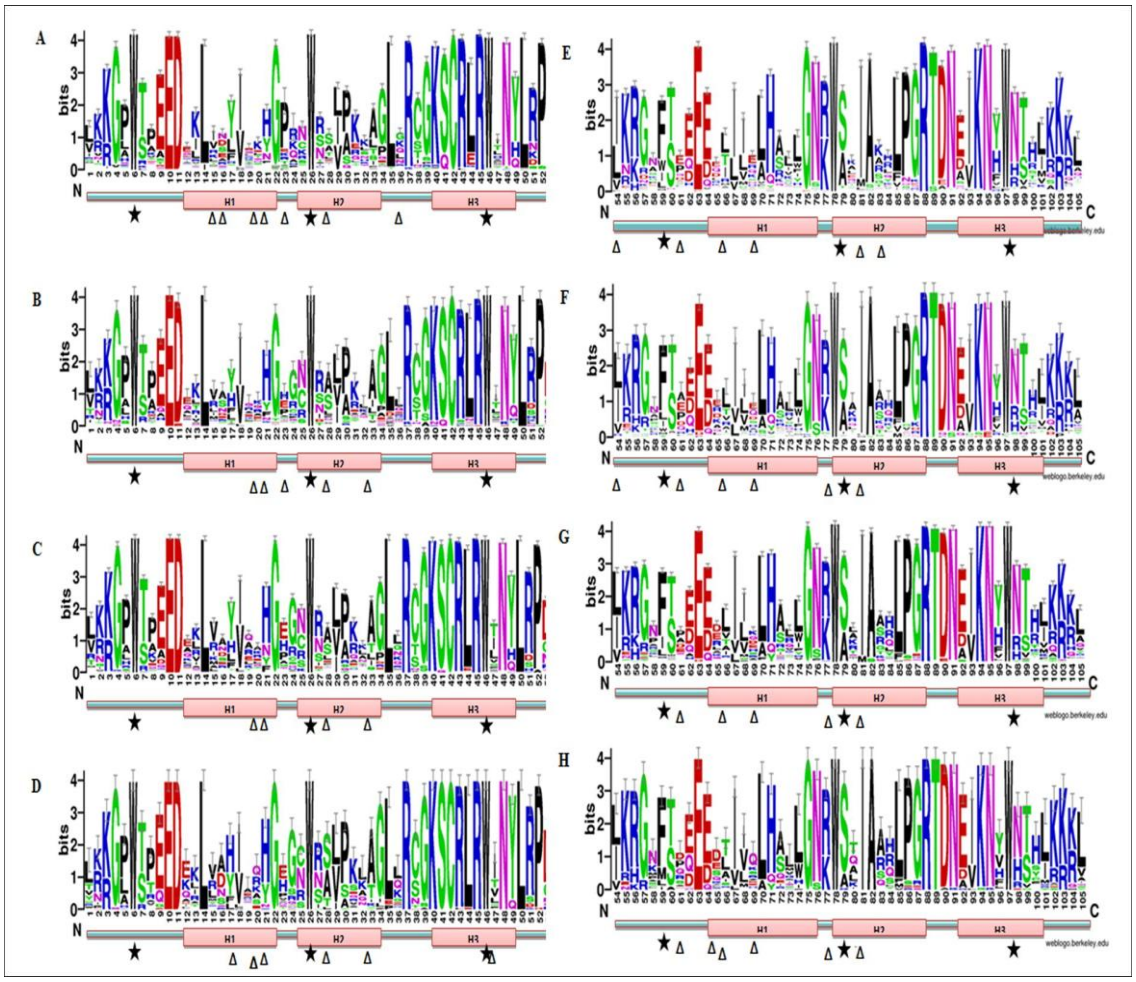

Figure 3.2 Comparisons of DNA binding domain of R2R3-MYB transcription factor proteins in *Arabidopsis*, *Brachypodium*, maize and barley. Logos A, B, C and D represent for the R2 repeat; E, F, G, H represent for the R3 repeat of R2R3-MYBs in *Arabidopsis*, *Brachypodium*, maize and barley, respectively. Helices are named as H1, H2 and H3 on each repeats. Tryptophan amino acids were labeled with asterisks, Triangle shows changed residues in the conserved domain of R2R3-MYBs.

3.1.3 Genome Distribution of Barley R2R3-type MYB TFs

Since the barley genome sequence has already been released yet, the chromosomal locations of MYBs were predicted by comparing with their *Brachypodium* homologues. Firstly, protein sequences of MYB proteins were blasted for *Brachypodium distachyon* (JGI/MIPS v1) by using phytozome search tool http://www.phytozome.net (Goodstein et al. 2012). Then, the highest scored one was taken as a candidate for barley protein homolog. Subsequently, the Bradi homologues were checked and filtered in the data published by Mayer et al. 2011. So, the putative chromosomal locations of barley MYB genes were predicted in the barley genome by

considering the *Brachypodium* and barley homology. As a result of predicting the chromosomal distributions of barley MYBs, it was determined that each of the chromosome (totally 7) included at least one R2R3-type MYB. In total, 51 R2R3 MYB transcription factor genes were distributed along with the chromosomes (6, 7, 5, 8, 13, 11 and 1, respectively) (Figure 3.3).. The highest R2R3 MYB density was observed in chromosome 5; and the lowest in chromosome 7. In general, MYBs were found not reside at the upper and lower parts of the chromosomes.

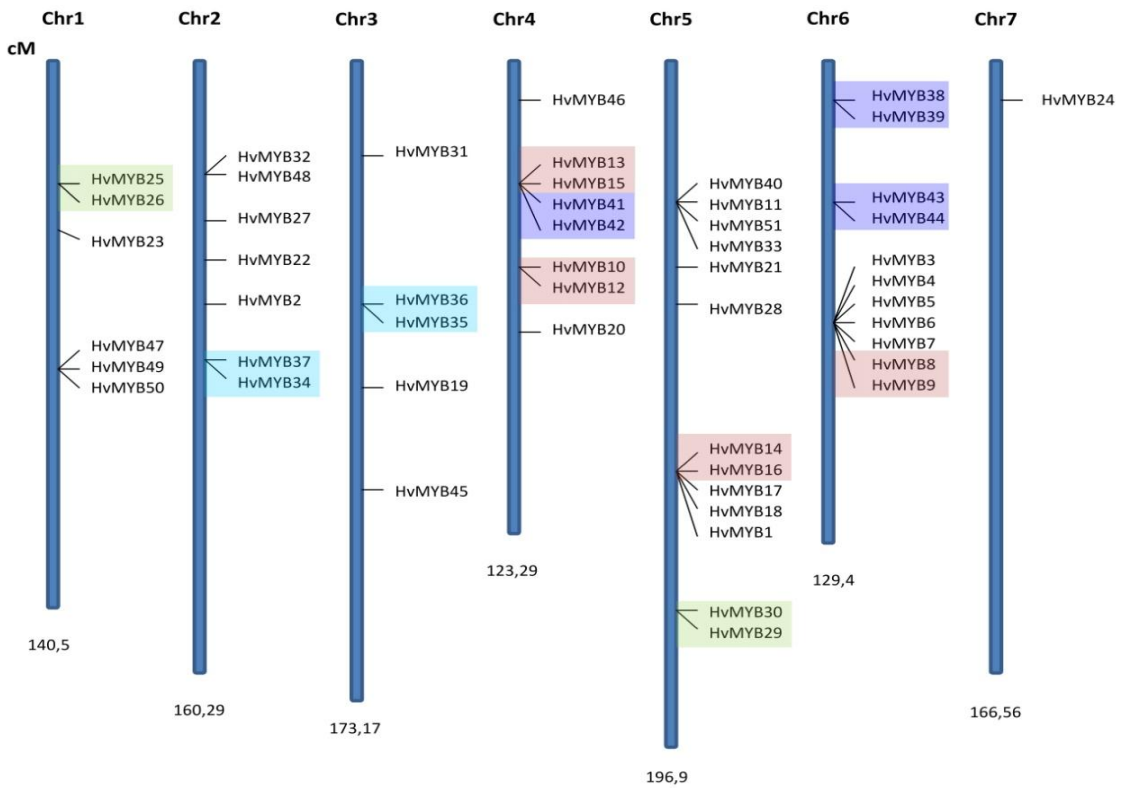

Figure 3.3 Genome distribution, duplication and predicted cluster of identified R2R3 MYB transcription factor genes in barley. Colored boxes show tandem and segmental duplications. The size of each chromosome is indicated as cM (centi-Morgan).

Beside the chromosomal distributions, gene duplications were also predicted among barley R2R3 MYB TFs. Duplications can occur as region-specific duplication or genome-wide polyploidization (Du et al. 2012b). They can be important reason for genome expansion and it increases the number of gene numbers that may gain new functions to an organism. 51 R2R3 MYB transcription factors were found to be

dispersed in the genome and putative tandem and segmental duplications were observed (Figure 3.3). Among these, 22 of R2R3 MYBs were predicted to be involved in duplication event. Segmental duplications were resided in 11 different locations. Based on the sequence similarity, they were clustered into four classes which were shown in colored boxes (Figure 3.3). Accordingly, the first cluster included HvMYB24: HvMYB25 and HvMYB29: HvMYB30; the second included HvMYB34: HvMYB37 and HvMYB36: HvMYB35; the third included HvMYB13: HvMYB15, HvMYB10: HvMYB12, HvMYB14: HvMYB16 and HvMYB8: HvMYB9; and the fourth included HvMYB41: HvMYB42, HvMYB43: HvMYB44 and HvMYB38: HvMYB39 (Figure 3.3).

3.1.4 Comparative Phylogenetic Analysis of R2R3 MYB Family Proteins in Barley and *Arabidopsis*

Phylogenetic trees were constructed separately for the DBD and full protein sequences of R2R3 MYB proteins (Figure 3.4; Figure 3.5). In addition, comparative phylogenetic analysis was conducted with barley and *Arabidopsis* MYB TFs (Figure 3.6). Neighbour joining (NJ) method was used to compute statistical distances within the R2R3 MYBs. In this study, combining the data from *Arabidopsis*, the number of subgroups was expanded to 34 (S1 to S34) (Figure 3.6). The identified 51 R2R3-type TFs were found dispersed within the 18 MYB subgroups. S26, S27 and S30 were composed of only barley MYBs other than the *Arabidopsis*. Beside, some of the barley MYBs were collectively been gathered and expanded the *Arabidopsis* subgroups. For instance, six barley MYBs (HvMYB-2, -7, -8, -10, -17 and -38) were placed in subgroup 28. Others were found to be dispersed within the overall barley subgroups (Figure 3.6).

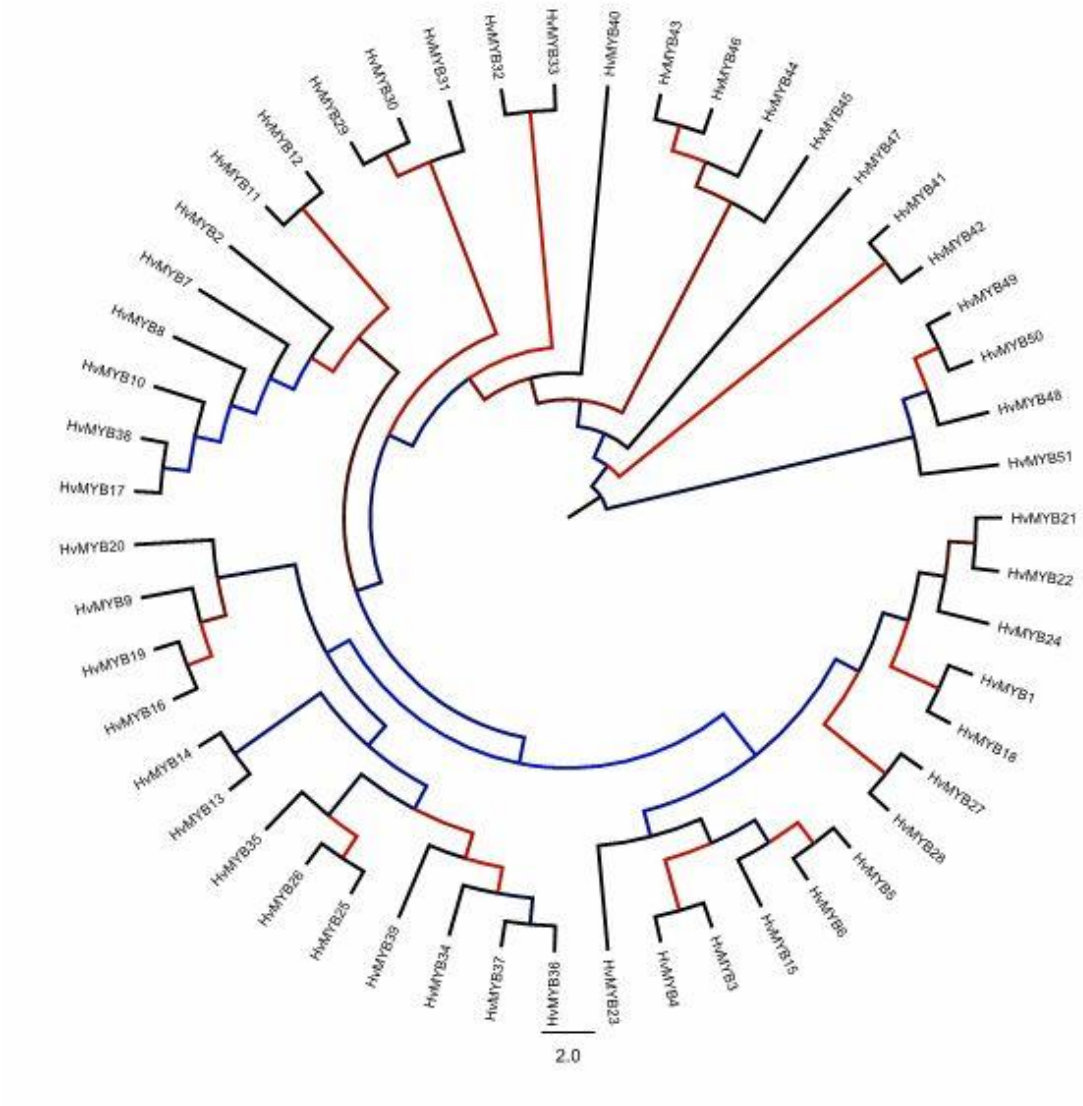

Figure 3.4 Phylogenetic analysis of 51 DBD of MYBs in barley.

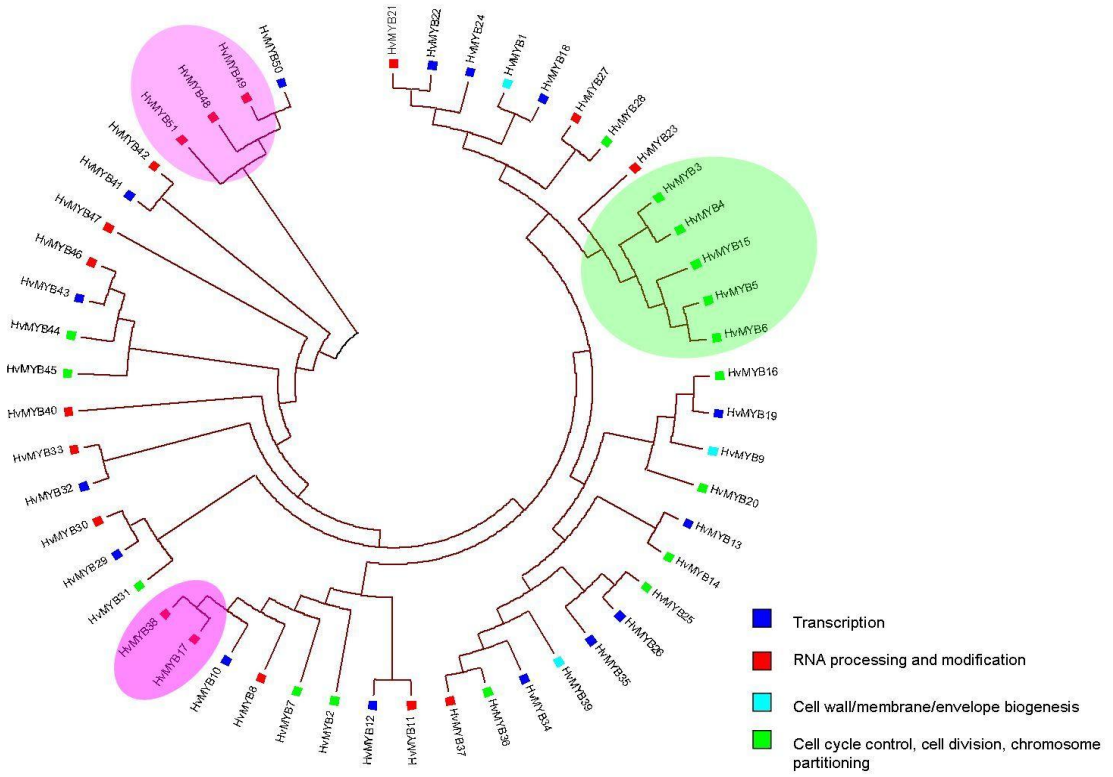

Figure 3.5 Phylogenetic analysis of 51 R2R3 MYB TFs in barley. NJ tree was constructed and TFs were functionally grouped into four classes according to COG classification method. Filled circles indicate functionally same TFs that were located at the same taxon. Also, they were found at the same chromosomal location in the genome (Figure 3.3).

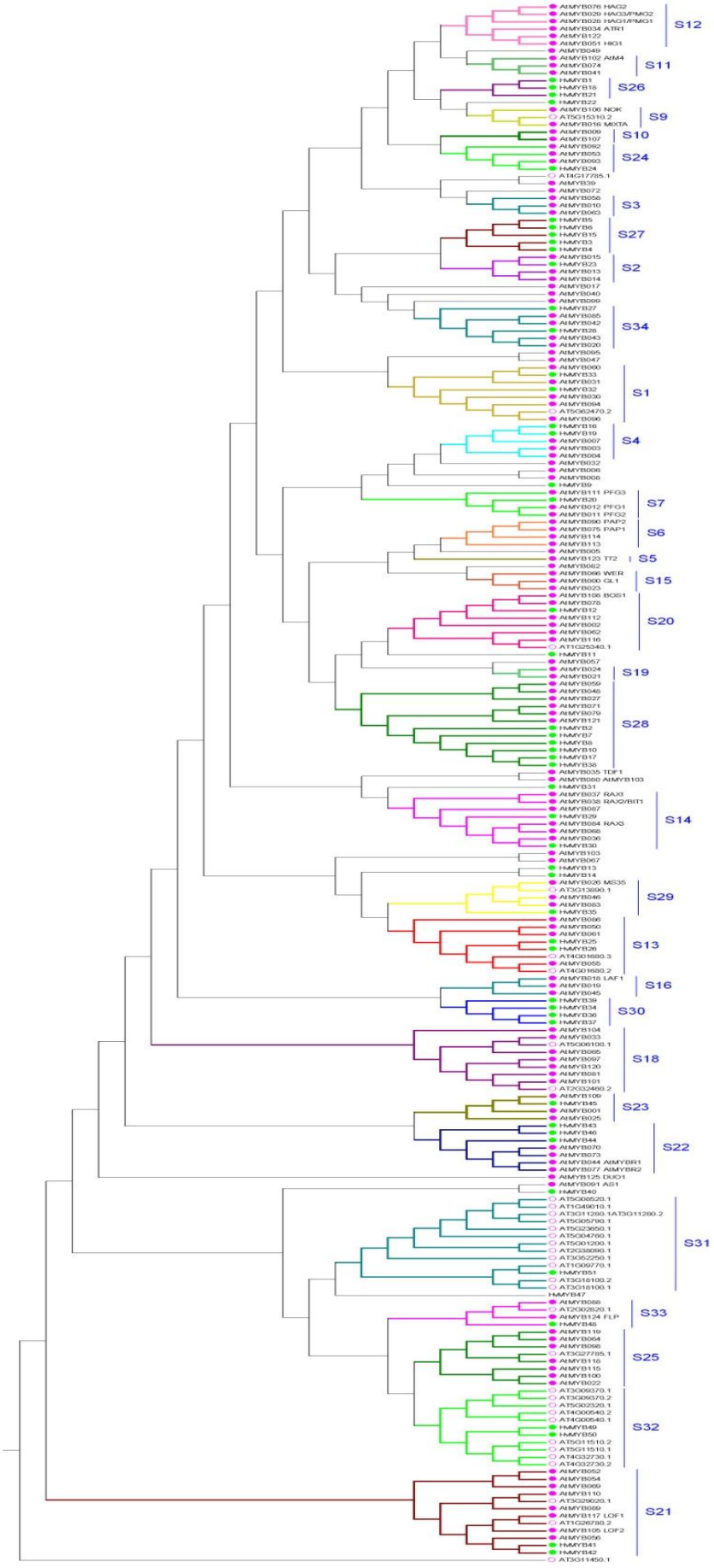
AtMYB076 HAG2
AtMYB029 HAG3/PMG2
AtMYB028 HAG1/PMG1
AtMYB034 ATR1
AtMYB122
AtMYB051 HIG1
AtMYB049
AtMYB102 AtM4
AtMYB074
AtMYB041
HMYB1
HMYB16
HMYB21
HMYB22
AtMYB106 NOK
AT5G15310.2
AtMYB016 MIXTA
AtMYB006
AtMYB107
AtMYB092
AtMYB053
AtMYB093
HMYB24
AT4G17785.1
AtMYB39
AtMYB072
AtMYB058
AtMYB010
AtMYB063
HMYB5
HMYB6
HMYB15
HMYB3
HMYB4
AtMYB015
HMYB23
AtMYB013
AtMYB014
AtMYB017
AtMYB040
AtMYB099
HMYB27
AtMYB085
AtMYB042
HMYB28
AtMYB043
AtMYB020
AtMYB095
AtMYB047
AtMYB060
HMYB33
AtMYB031
HMYB32
AtMYB030
AtMYB094
AT5G62470.2
AtMYB096
HMYB16
HMYB19
AtMYB007
AtMYB003
AtMYB004
AtMYB032
AtMYB005
AtMYB008
HMYB9
AtMYB111 PFG3
HMYB20
AtMYB012 PFG1
AtMYB011 PFG2
AtMYB090 PAP2
AtMYB075 PAP1
AtMYB114
AtMYB113
AtMYB005
AtMYB123 TT2
AtMYB082
AtMYB086 WER
AtMYB000 GL1
AtMYB023
AtMYB108 BOS1
AtMYB078
HMYB12
AtMYB112
AtMYB052
AtMYB062
AtMYB116
AT1G25340.1
HMYB11
AtMYB057
AtMYB024
AtMYB021
AtMYB059
AtMYB046
AtMYB027
AtMYB071
AtMYB079
AtMYB121
HMYB2
HMYB7
HMYB8
HMYB10
HMYB17
HMYB38
AtMYB035 TDF1
AtMYB080 AtMYB103
HMYB31
AtMYB037 RAX1
AtMYB038 RAX2/BIT1
AtMYB067
HMYB29
AtMYB084 RAX3
AtMYB068
AtMYB036
HMYB30
AtMYB103
AtMYB067
HMYB13
HMYB14
AtMYB026 MS35
AT3G13890.1
AtMYB046
AtMYB083
HMYB35
AtMYB066
AtMYB050
AtMYB061
HMYB25
HMYB26
AT4G01680.3
AtMYB055
AT4G01680.2
AtMYB018 LAF1
AtMYB019
AtMYB045
HMYB39
HMYB34
HMYB36
HMYB37
AtMYB104
AtMYB033
AT5G06100.1
AtMYB065
AtMYB097
AtMYB120
AtMYB081
AtMYB101
AT2G32460.2
AtMYB109
HMYB45
AtMYB001
AtMYB025
HMYB43
HMYB46
HMYB44
AtMYB070
AtMYB073
AtMYB044 AtMYBR1
AtMYB077 AtMYBR2
AtMYB125 DUO1
AtMYB091 AS1
HMYB40
AT5G08520.1
AT1G49010.1
AT3G11280.1AT3G11280.2
AT5G05790.1
AT5G23650.1
AT5G04760.1
AT5G01200.1
AT2G38090.1
AT3G52250.1
AT1G09770.1
HMYB51
AT3G16100.2
AT3G16100.1
HMYB47
AtMYB088
AT2G02820.1
AtMYB124 FLP
HMYB48
AtMYB119
AtMYB064
AtMYB098
AT3G27785.1
AtMYB116
AtMYB115
AtMYB103
AtMYB022
AT3G06370.1
AT3G06370.2
AT5G02320.1
AT4G00540.2
AT4G00540.1
HMYB49
HMYB50
AT5G11510.2
AT5G11510.1
AT4G32730.1
AT4G32730.2
AtMYB052
AtMYB054
AtMYB069
AtMYB110
AT3G29020.1
AtMYB089
AtMYB117 LOF1
AT1G26780.2
AtMYB105 LOF2
AtMYB056
HMYB41
HMYB42
AT3G11450.1
S12
S11
S26
S9
S10
S24
S3
S27
S2
S34
S1
S4
S7
S6
S5
S15
S20
S19
S28
S14
S29
S13
S16
S30
S18
S23
S22
S31
S33
S25
S32
S21

Figure 3.6 Comparative phylogenetic analysis of barley R2R3 MYB TFs with *Arabidopsis*. In total, they were fall into 34 subgroups. *Arabidopsis* and barley MYBs were labeled with colored spots: *Hv*MYBs green and *At*MYBs pink in color. Circles represent putative *At*MYBs.

By using COG (Clusters of Orthologous Groups) classification method, 51 barley R2R3 MYB TFs were grouped according to their functional categories. Among 25 COG categories, 51 barley MYB proteins were fell into four distinct classes including: cell cycle control, cell division, chromosome partitioning (16), cell wall/membrane/envelope biogenesis (3), RNA processing and modification (17), transcription (15) (Table 3.1). Also functional categories of barley MYB proteins were separately shown by using a NJ tree (Figure 3.5). The distinct classes were labeled for each barley MYB proteins.

Table 3.1 Functional-categories of identified R2R3 MYB transcription factors in barley.

MYB proteins	Accession Numbers***	Functional-Categories*	Function**	Group
HvMYB01	SAMN01915685	Cell wall/membrane/envelope biogenesis		
HvMYB02	SAMN01915686	Cell cycle control, cell division, chr partitioning		
HvMYB03	SAMN01915687	Cell cycle control, cell division, chr partitioning		
HvMYB04	SAMN01915688	Cell cycle control, cell division, chr partitioning		
HvMYB05	SAMN01915689	Cell cycle control, cell division, chr partitioning		
HvMYB06	SAMN01915690	Cell cycle control, cell division, chr partitioning		
HvMYB07	SAMN01915691	Cell cycle control, cell division, chr partitioning		
HvMYB08	SAMN01915692	RNA processing and modification		
HvMYB09	SAMN01915693	Cell wall/membrane/envelope biogenesis		
HvMYB10	SAMN01915694	Transcription		
HvMYB11	SAMN01915695	RNA processing and modification		
HvMYB12	SAMN01915696	Transcription	Defense, development	S20
HvMYB13	SAMN01915697	Transcription		
HvMYB14	SAMN01915698	Cell cycle control, cell division, chr partitioning		
HvMYB15	SAMN01915699	Cell cycle control, cell division, chr partitioning		
HvMYB16	SAMN01915700	Cell cycle control, cell division, chr partitioning	Metabolism	S4
HvMYB17	SAMN01915701	RNA processing and modification		
HvMYB18	SAMN01915702	Transcription		
HvMYB19	SAMN01915703	Transcription	Metabolism	S4
HvMYB20	SAMN01915704	Cell cycle control, cell division, chr partitioning	Metabolism	S7
HvMYB21	SAMN01915705	RNA processing and modification		
HvMYB22	SAMN01915706	Transcription		
HvMYB23	SAMN01915707	RNA processing and modification	Defense	S2

Table 3.1 Functional-categories of identified R2R3 MYB transcription factors in barley (Continued).

HvMYB24	SAMN01915708	Transcription		S24
HvMYB25	SAMN01915709	Cell cycle control, cell division, chr partitioning	Metabolism	S13
HvMYB26	SAMN01915710	Transcription	Metabolism	S13
HvMYB27	SAMN01915711	RNA processing and modification		
HvMYB28	SAMN01915712	Cell cycle control, cell division, chr partitioning		
HvMYB29	SAMN01915713	Transcription	Development	S14
HvMYB30	SAMN01915714	RNA processing and modification	Development	S14
HvMYB31	SAMN01915715	Cell cycle control, cell division, chr partitioning		
HvMYB32	SAMN01915716	Transcription	Defense, development	S1
HvMYB33	SAMN01915717	RNA processing and modification	Defense, development	S1
HvMYB34	SAMN01915718	Transcription		
HvMYB35	SAMN01915719	Transcription		
HvMYB36	SAMN01915720	Cell cycle control, cell division, chr partitioning		
HvMYB37	SAMN01915721	RNA processing and modification		
HvMYB38	SAMN01915722	RNA processing and modification		
HvMYB39	SAMN01915723	Cell wall/membrane/envelope biogenesis		
HvMYB40	SAMN01915724	RNA processing and modification		
HvMYB41	SAMN01915725	Transcription	Metabolism, development	S21
HvMYB42	SAMN01915726	RNA processing and modification	Metabolism, development	S21
HvMYB43	SAMN01915727	Transcription		
HvMYB44	SAMN01915728	Cell cycle control, cell division, chr partitioning		
HvMYB45	SAMN01915729	Cell cycle control, cell division, chr partitioning		S23
HvMYB46	SAMN01915730	RNA processing and modification		
HvMYB47	SAMN01915731	RNA processing and modification		
HvMYB48	SAMN01915732	RNA processing and modification		
HvMYB49	SAMN01915733	RNA processing and modification		
HvMYB50	SAMN01915734	Transcription		
HvMYB51	SAMN01915735	RNA processing and modification		

* Functional categories were obtained from annotation data of full transcriptome profiling of barley. Among 25 categories, MYBs fell into four groups.
** Function series were adapted from the supplementary document published by Dubos et al. 2010.
*** Accession numbers were obtained as the result of gene submission to NCBI database.

3.1.5 Expressions of R2R3-type MYB family proteins under Boron stress

In order to detect the expression levels of MYB genes in control and Boron stressed conditions (1000µM Root, Leaf), RPKM (Reads Per Kilobase per Million mapped reads) values were compared with the control groups (50µM Root; 50µM Leaf). RPKM is defined as a quantification method for gene expressions by the data

obtained from RNA sequencing, in order to normalize for total read length and the number of sequencing reads (Mortazavi et al. 2008). In Figure 3.7, RPKM values were represented for each identified MYB proteins. It was clearly observed that some MYBs were only expressed in root or in leaf tissues for control (50μM) and B-stressed (1000 μM) samples. For instance, MYB15, 24, 29, 30, 36, 37, 41 and 47 were expressed only in root; MYB20, 32 and 33 were expressed only in leaf. Additionally, no transcript accumulation was observed for some MYB genes. For instance, MYB2, 3, 4, 5, 6 and 38 was not detected in both control and B-stressed root and leaf tissues. These genes may be expressed at specific conditions or any of specific developmental stages in plant. In the Figure 6, RPKM changes were shown between the control and the subject groups. According to the data, 25 of the 51 MYB TFs (~49%) were down-regulated in 1000 μM B exposed roots (50R/1000R). In the 9 (~18%) TFs no expression change was observed. Beside, 17 MYB TFs (%33) were found up-regulated in B induced barley roots. In the leaf samples, B induction affected to MYB gene expressions as; 51 of 19 (~%37) induced; 13 (~%25) repressed and 19 (~%37) showed no expression change.

	ROOT		LEAF	
	50 RPKM	1000 RPKM	50 RPKM	1000 RPKM
HvMYB01	6,76	6,34	0,53	3,69
HvMYB02	0,00	0,00	0,00	0,00
HvMYB03	0,00	0,00	0,00	0,00
HvMYB04	0,00	0,00	0,00	0,00
HvMYB05	0,00	0,00	0,00	0,00
HvMYB06	0,00	0,00	0,00	0,00
HvMYB07	4,38	4,25	0,27	0,11
HvMYB08	0,00	0,00	0,00	0,00
HvMYB09	5,76	3,97	0,15	0,30
HvMYB10	0,95	0,71	0,04	0,10
HvMYB11	6,83	4,66	0,26	0,86
HvMYB12	4,94	5,34	0,27	0,00
HvMYB13	4,73	5,86	0,34	0,26
HvMYB14	7,26	8,00	5,28	3,77
HvMYB15	5,21	0,80	0,00	0,00
HvMYB16	6,91	7,49	5,05	5,65
HvMYB17	0,26	0,10	0,00	0,09
HvMYB18	5,86	6,05	0,53	0,46
HvMYB19	6,56	6,29	0,30	0,72
HvMYB20	0,00	0,02	5,10	3,40
HvMYB21	4,36	4,03	0,49	0,94
HvMYB22	5,86	5,33	4,01	5,14
HvMYB23	6,10	6,24	0,84	0,26
HvMYB24	5,04	5,47	0,00	0,00
HvMYB25	4,41	3,97	0,18	0,10
HvMYB26	4,81	4,21	0,36	0,00
HvMYB27	5,13	4,44	0,19	0,42
HvMYB28	4,88	4,60	0,12	0,37
HvMYB29	4,90	5,15	0,00	0,00
HvMYB30	7,61	7,56	0,00	0,00
HvMYB31	7,30	7,16	4,34	5,81
HvMYB32	0,18	0,09	0,60	4,84
HvMYB33	0,00	0,00	6,10	6,72
HvMYB34	0,00	0,14	0,00	0,00
HvMYB35	5,89	6,80	0,18	0,07
HvMYB36	5,72	6,30	0,00	0,05
HvMYB37	3,99	0,90	0,08	0,00
HvMYB38	0,00	0,00	0,00	0,00
HvMYB39	6,07	5,45	0,00	0,00
HvMYB40	0,08	0,35	6,17	0,00
HvMYB41	5,59	3,90	0,02	0,00
HvMYB42	5,11	6,22	0,62	0,54
HvMYB43	6,72	6,22	4,17	3,58
HvMYB44	7,42	7,19	5,28	5,90
HvMYB45	4,07	3,91	0,68	0,82
HvMYB46	3,52	3,97	0,23	0,26
HvMYB47	8,30	7,35	0,00	0,00
HvMYB48	6,84	6,93	5,26	5,24
HvMYB49	4,54	4,85	0,54	3,57
HvMYB50	0,41	0,90	0,16	0,26
HvMYB51	7,77	7,86	6,16	6,85

Figure 3. 7 Expressions of 51 barley R2R3 MYB-type transcription factors in control (50 µM) and B-stressed (1000 µM) root and leaf tissues. The number indicated on each cell represents the log2 calculated RPKM values. RPKM values smaller than 1 was not calculated due to negative logarithm and they were stated as in the original data.

3.2 QUANTITATIVE ANALYSIS OF PHLOEM-DERIVED Cuc-miRNAs UNDER BORON STRESS

In this section, expressions of 14 miRNA (miR156, miR159, miR160, miR162, miR166, miR167, miR168, miR169, miR172, miR390, miR393, miR397, and miR398) were analyzed by using qRT-PCR experiments. To test contamination during sample collection, an experiment was designed including with a positive and a negative control genes.

3.2.1 Contamination Test

In this experiment, the most important and critical point in the initial steps of the experiment was collecting the PS (phloem sap) from pumpkins without contamination. There are a few methods for PS harvesting which are proposed by several researches. In here, the PS exudation was simply carried out by spontaneous leakage of the sap from the cut surface. The method can be simply defined as “cut and wait”. The term was firstly proposed in here. In spite of its efficiency, the method can be problematic due to the contamination risks during PS exudation. To eliminate the contaminations, first droplets of PS need to be removed from the cut surface with a sterile filter paper. After this step, the exudates can be collected in a pre-cooled tube. Nevertheless, a contamination test is essential after the PS collection to make certain of its purity. To test it, an experiment was designed and carried out. Accordingly, two genes (*CmPP16* and *RuBisCo*) were used for the PS RNAs as positive and negative control groups, respectively.

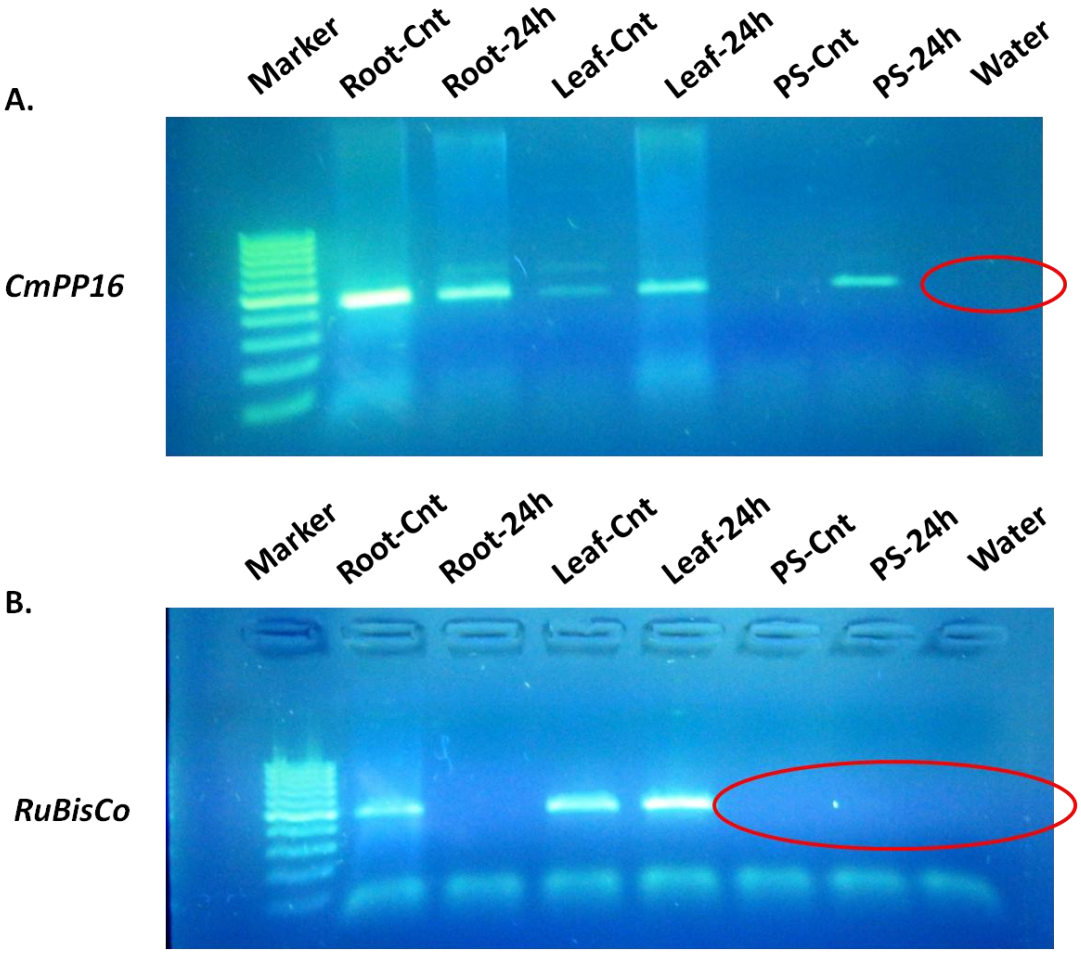

Figure 3.8 Gels represented above shows the contamination test results. *CmPP16* gene was used as a positive control which it must be found in all tissues (A). Also, pumpkin specific *RuBisCo* gene was amplified to test whether there was a contamination during phloem sap (PS) exudation (B). In all reactions, water was added to the reaction mixture instead of a cDNA template (water) and used as a negative control. A 100 bp Marker (M) (Thermo) was used to predict the size distributions of bands. RNA isolations and cDNA synthesis were carried out from the leaf (L), root (R), and PS exudates under conditions of control (Cnt) and B-exposed ones (24h).

CmPP16 gene was used as a positive control which it must be found in all type of tissues including PS. Also, pumpkin specific *RuBisCo* gene was amplified to test whether there was a contamination during phloem sap (PS) exudation. Normally plant tissues are involved RuBisCo protein, however PS do not. For the gene amplification reactions, water was added to the reaction mixture instead of a cDNA template (water) and they were used as negative controls. 100 bp Marker (M) (Thermo) was used to

predict the size distributions of bands. RNA isolations and cDNA synthesis were carried out from the leaf (L), root (R), and PS exudates under conditions of control (Cnt) and B-exposed ones (24h). According to the results, it was observed that PS samples were included the *CmPP16* transcripts. However, the *RuBisCo* gene was not amplified in both Control and 24h B-exposed PS samples (Figure 3.12). These results clearly indicated that during the PS exudation and RNA isolation processes the samples are well protected from the tissue artefacts. In negative control reactions, no amplification was observed and it means that our experiments reliable and suitable for the next qPCR analysis (Figure 3.8).

3.2.2 RT-qPCR Analysis of phloem-derived miRNAs

miRNAs are key players in plants during the signal transduction over long distances. In here, 14 miRNA transcripts were searched in the PS of control and B-stressed pumpkin plants. After the contamination tests, we clarified that our samples were avoided from non-phloem RNAs and we subsequently carried out quantitative expression experiments. For this aim, first strand cDNA of each miRNA were synthesized by using miRNA specific primers following the quantitative expression analysis. In here, it is noteworthy to say that the amount of RNA used for the cDNA synthesis was 30 ng for each PS samples. Indeed, it was a risk for the experiment because the amount of template RNA was so limited due to deficiency of the starting material (PS). Nevertheless, the experiments were carried out and we got efficient results. The qPCR analysis was carried out with two technical replications.

According the results, all of selected miRNA were found to be resided in the phloem sap of *C. maxima* plants. In addition, some miRNA molecules were found to be over-expressed against B-induction and as an early result, we propose that those miRNAs may be correlated with B stress and acts as a B-responsive signal molecule. For instance in the quantitation analysis, it was found that the accumulations of Cma-miR156, Cma-miR160, Cma-miR166, Cma-miR169, Cma-miR172, and Cma-miR393 were increased against B-induction. When we compared the relative expressions some miRNAs were found to be really over-expressed even some miRNA expressions were seem to be B-stress dependent. For instance, Cma-miR156, Cma-miR160, Cma-miR166, and Cma-miR172 were regularly resided in PS of control plants. However, Cma-miR169 and Cma-miR393 expressions were observed only in B-stress induced

plants. This result indicated that Cma-miR156, Cma-miR160, Cma-miR166, and Cma-miR172 were slightly responded against B-stress. However, Cma-miR169 and Cma-miR393 were highly accumulated in the PS of pumpkin against B-stress.

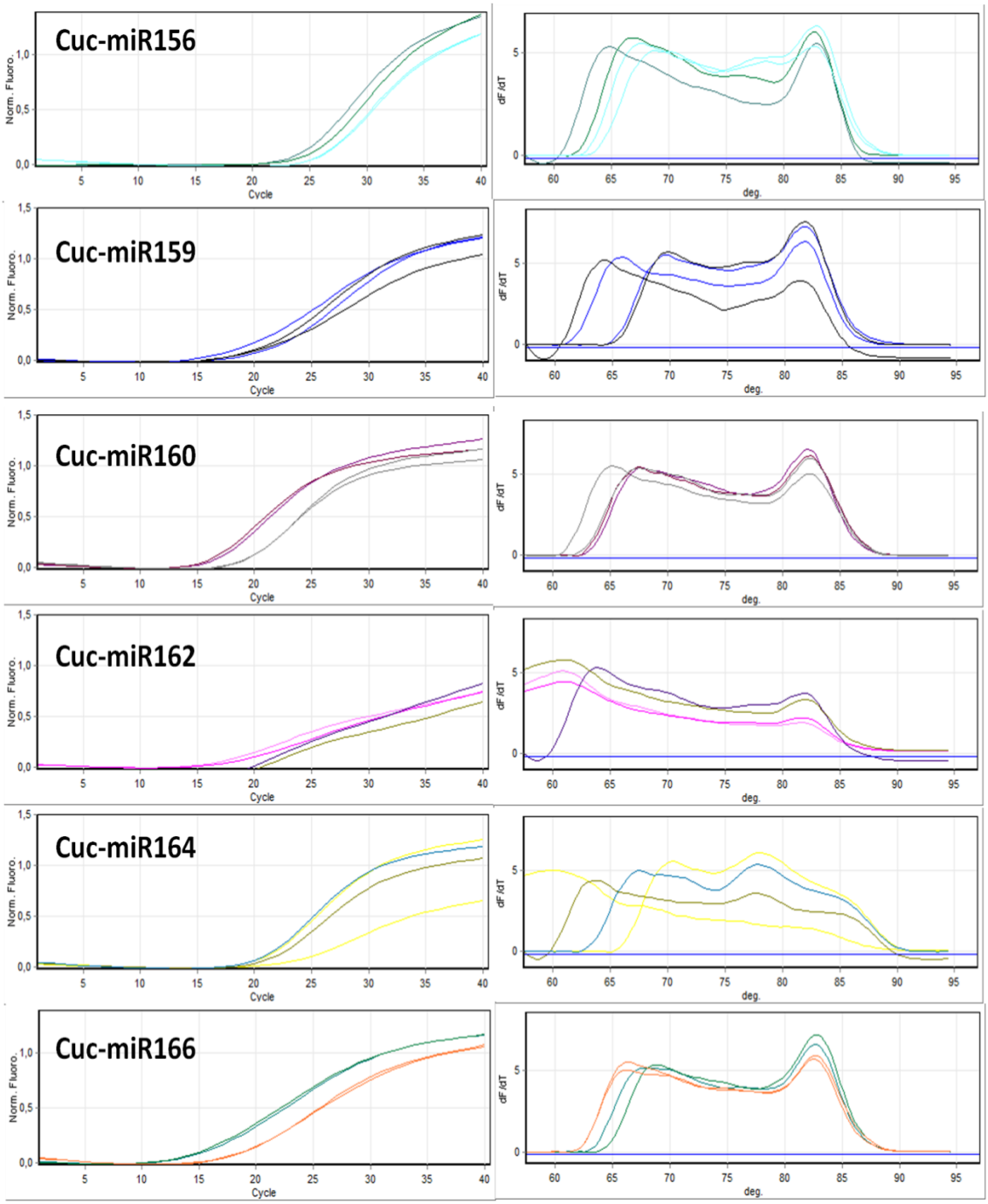

Figure 3.9 Quantitative analysis of phloem derived Cma-miRNAs under normal and B-stress conditions. miR156, miR160, miR166 seem to be expressed against B-stress. The graphs represent the quantitation (left-hand side) and melting curve (right-hand side) analysis of Cma-miRNAs.

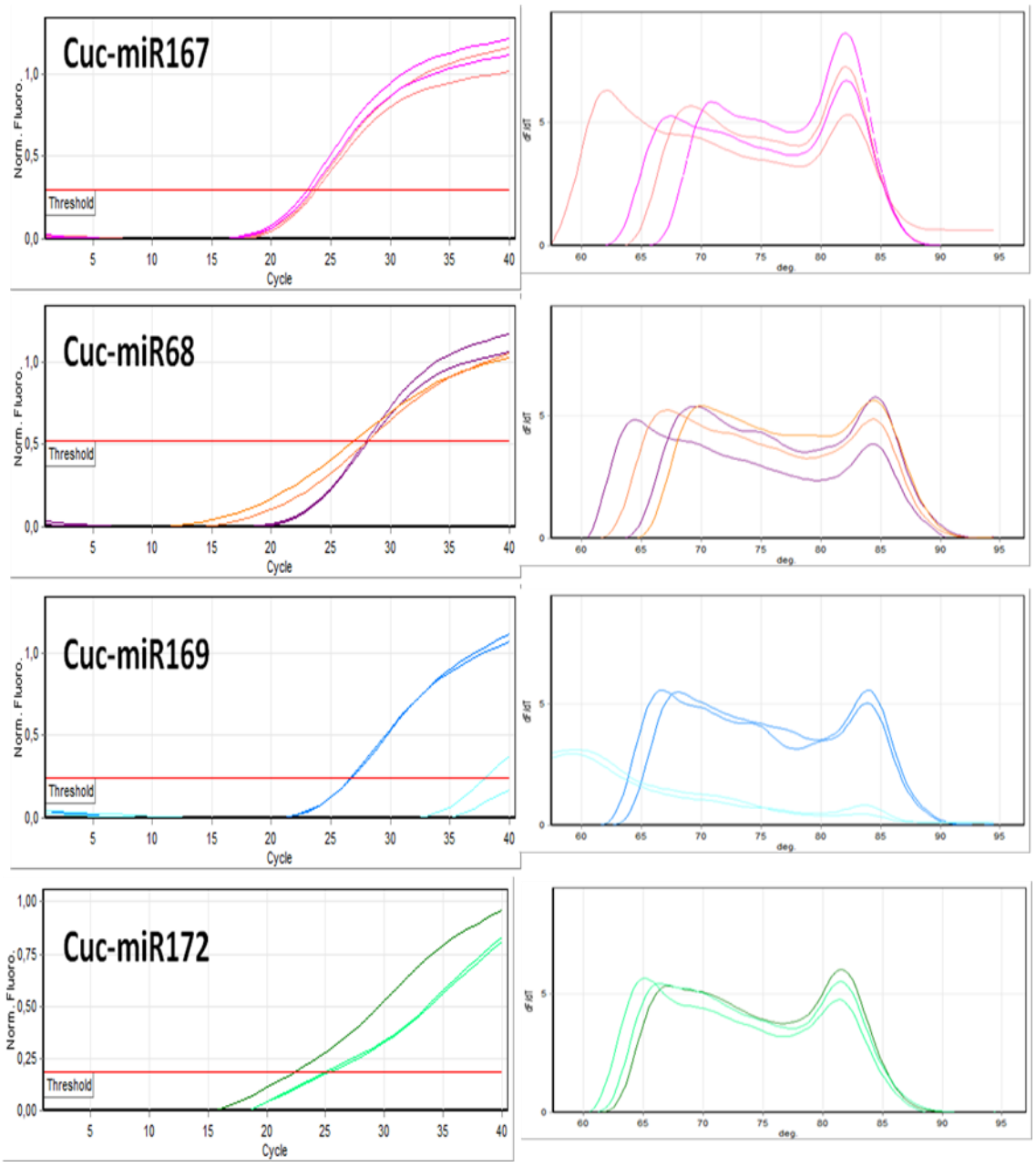

Figure 3.10 Quantitative analysis of phloem derived Cma-miRNAs under normal and B-stress conditions. In here, miR167, miR168, miR169, miR172 expressions were observed in qRT PCR analysis. Melting curve analysis shows that miRNA-spesific primers were targeted to their corresponding miRNA sequences. miR172 and miR169 showed an expression stimulation against B-stress. The graphs represent the quantitation (left-hand side) and melting curve (right-hand side) analysis of Cma-miRNAs.

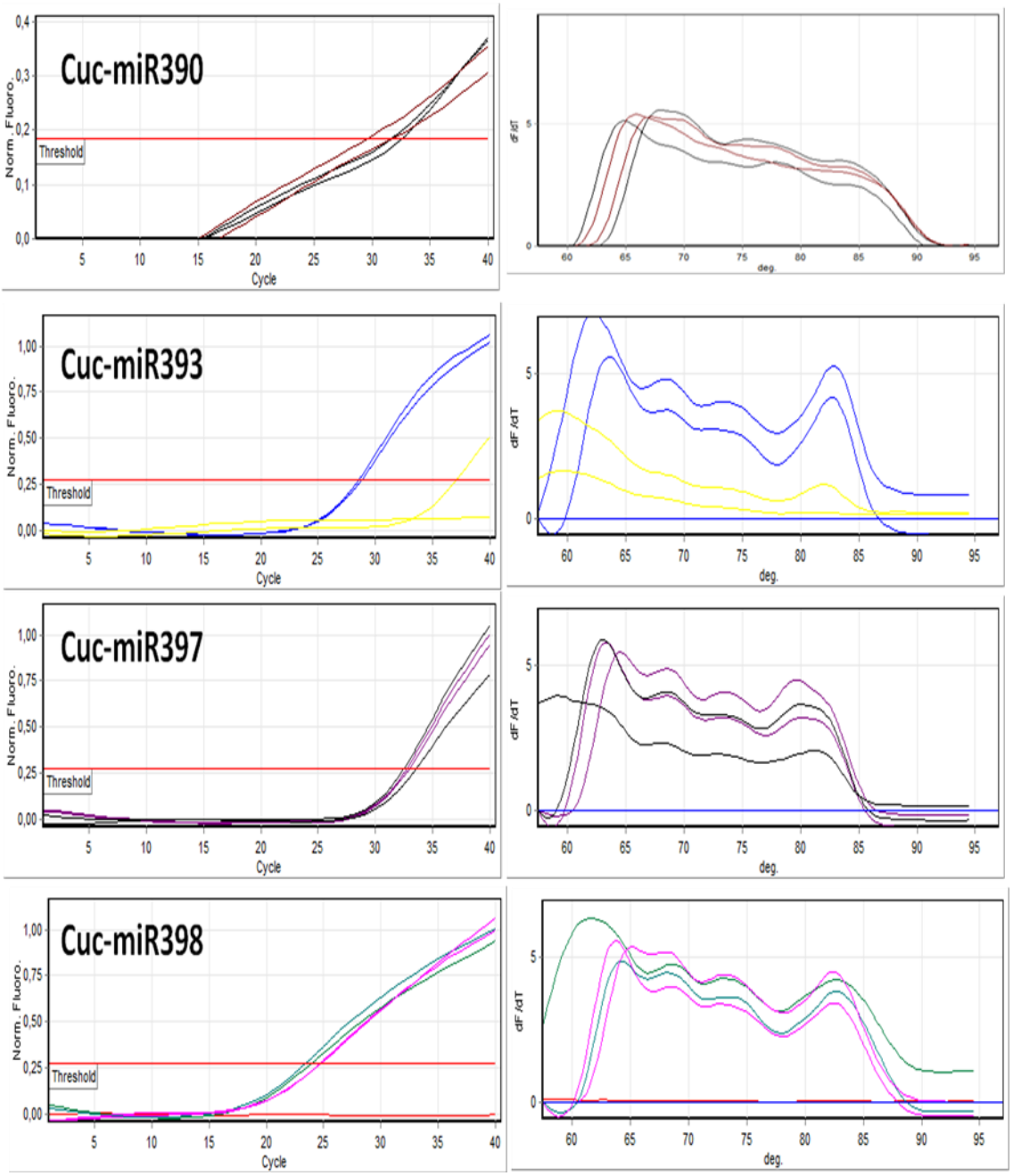

Figure 3.11 Quantitative analysis of phloem derived Cma-miRNAs under normal and B-stress conditions. In here, miR390, miR393, miR397, and miR398 expressions were observed in qRT PCR analysis. Melting curve analysis shows that miRNA-spesific primers were targeted to their corresponding miRNA sequences. miR393 showed an expression stimulation against B-stress. miR390 gave a negative signal against primer specificity. The graphs represent the quantitation (left-hand side) and melting curve (right-hand side) analysis of Cma-miRNAs.

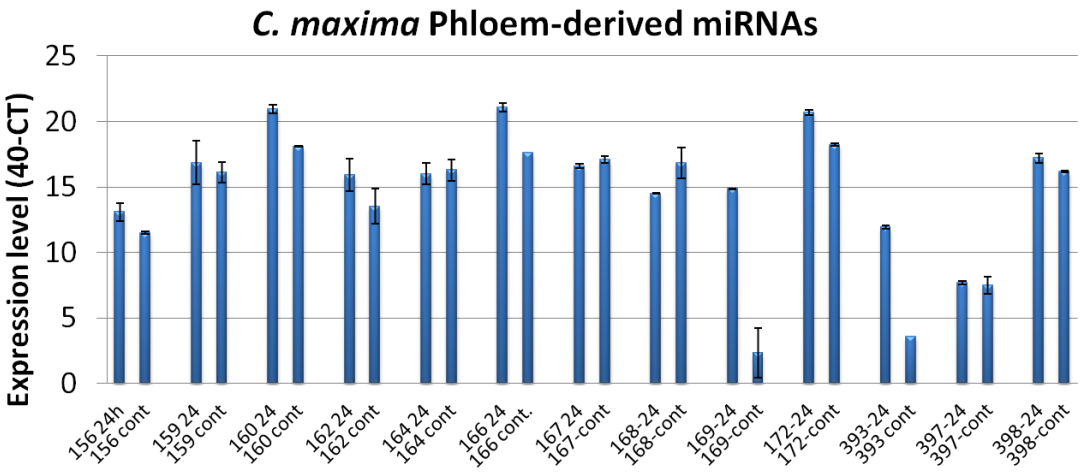

Figure 3.12 miRNA expression profiles of phloem sap of control and B-exposed plants. The expressions were calculated according the Ct values of each sample. Standard deviations were formulated by considering the two technical replicates.

According to stem-loop RT-qPCR analysis of *C. maxima* phloem miRNAs, we found that 13 of 9 miRNAs were found to be over-expressed against B-induction. In addition, 4 miRNA expressions were slightly decreased. Among the up-regulated ones, miR169 and miR393 were highly up-regulated. This results show that some miRNA molecules are activated against B-induction and they may be involve in the long distance communication of plants under B-stress condition. Especially miR169 and miR393 may be critical to sense and respond to the B-stress.

To find the possible miRNA targets, we searched the miRNA sequences by using psRNATarget (Plant Small RNA Target Analysis Server) server in the "User submitted small RNAs preloaded samples" platform (http://plantgrn.noble.org/psRNATarget/). The results were given in Table 3.2.

Table 3.2 Possible miRNA targets in *C. maxima*.

miRNA	Target_Acc.	Target analysis	miRNA_aligned_fragment
MIR156	Csa1M015680.1	squamosa binding domain	UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M039890.1	*Cucumis sativus* uncharacterized LOC101225600 (LOC101225600)	UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M039890.3		UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M039890.2		UGACAGAAGAGAGUGAGCAC
MIR156	Csa3M809420.1	*Cucumis sativus* squamosa promoter-binding-like protein 17-like (LOC101203301)	UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M001450.1	squamosa promoter-binding-like protein 13A-like	UGACAGAAGAGAGUGAGCAC
MIR156	Csa3M117960.1		UGACAGAAGAGAGUGAGCAC
MIR156	Csa6M094760.1	*Cucumis sativus* squamosa promoter-binding-like protein 16-like (LOC101217032)	UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M051590.1	*Cucumis sativus* squamosa promoter-binding-like protein 13A-like (LOC101221156)	UGACAGAAGAGAGUGAGCAC
MIR156	Csa1M051590.2	*Cucumis sativus* squamosa promoter-binding-like protein 13A-like (LOC101221156)	UGACAGAAGAGAGUGAGCAC
MIR156	Csa2M148430.1		UGACAGAAGAGAGUGAGCAC
MIR156	Csa3M567830.1		UGACAGAAGAGAGUGAGCAC
MIR159	Csa4M022940.1	*Cucumis sativus* transcription factor MYB29-like (LOC101214818)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa6M105150.1	*Cucumis sativus* uncharacterized LOC101219480 (LOC101219480)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa3M850670.1		UUUGGAUUGAAGGGAGCUCU
MIR159	Csa6M046470.1	*Cucumis sativus* transcription factor GAMYB-like (LOC101213434)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa4M628340.2		UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa1M077140.1		UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa4M628340.1		UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa6M405340.1	*Cucumis sativus* mannan endo-1,4-beta-mannosidase 6-like (LOC101227669)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa4M088740.2		UUUGGAUUGAAGGGAGCUCU
MIR159	Csa4M088740.1		UUUGGAUUGAAGGGAGCUCU
MIR159	Csa2M429010.1	*Cucumis sativus* benzyl alcohol O-benzoyltransferase-like (LOC101208513)	UUUGGAUUGAAGGGAGCUCU
MIR159	Csa4M083490.2	*Cucumis sativus* BES1/BZR1 homolog protein 4-like (LOC101230113)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa2M035350.1	*Cucumis sativus* transcription factor MYB86-like (LOC101216498)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa4M083490.1	*Cucumis sativus* BES1/BZR1 homolog protein 4-like (LOC101230113)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa7M062900.1	*Cucumis sativus* outer envelope protein 64, mitochondrial-like (LOC101219942)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa5M182630.1	*Cucumis sativus* telomere repeat-binding protein 3-like (LOC101231022)	UUUGGAUUGAAGGGAGCUCUA
MIR159	Csa5M182630.2		UUUGGAUUGAAGGGAGCUCUA
MIR160	Csa2M315390.1	*Cucumis sativus* auxin response factor 17-like (LOC101216118)	UGCCUGGCUCCCUGUAUGCCA

Table 3.2 Possible miRNA targets in *C. maxima* (Continued).

MIR160	Csa6M445210.1	*Cucumis sativus* auxin response factor 18-like (LOC101217039)	UGCCUGGCUCCCUGUAUGCCA
MIR160	Csa6M405890.1	*Cucumis sativus* auxin response factor 18-like (LOC101204568)	UGCCUGGCUCCCUGUAUGCC
MIR160	Csa6M405890.2	*Cucumis sativus* auxin response factor 18-like (LOC101204568)	UGCCUGGCUCCCUGUAUGCC
MIR160	Csa6M141390.1	*Cucumis sativus* auxin response factor 18-like (LOC101216500)	UGCCUGGCUCCCUGUAUGCC
MIR162	Csa3M116650.1	*Cucumis sativus* endoribonuclease Dicer homolog 1-like (LOC101219617)	UCGAUAA-ACCUCUGCAUCCAG
MIR162	Csa4M642290.1	*Cucumis sativus* isoflavone 2'-hydroxylase-like (LOC101223426)	UCGAUAAACCUCUGCAUCCAG
MIR162	Csa4M642290.3		UCGAUAAACCUCUGCAUCCAG
MIR162	Csa4M642290.2		UCGAUAAACCUCUGCAUCCAG
MIR164	Csa4M629480.1	*Cucumis sativus* protein CUP-SHAPED COTYLEDON 2-like (LOC101205357)	UGGAGAAGCAGGGCACGUGCU
MIR164	Csa3M523580.1	*Cucumis sativus* NAC domain-containing protein 21/22-like (LOC101211941)	UGGAGAAGCAGGGCACGUGCU
MIR164	Csa1M038340.1	*Cucumis sativus* NAC domain-containing protein 100-like (LOC101220421)	UGGAGAAGCAGGGCACGUGC
MIR164	Csa3M824990.1	*Cucumis sativus* NAC domain-containing protein 100-like (LOC101214698)	UGGAGAAGCAGGGCACGUGC
MIR164	Csa1M009870.1		UGGAGAAGCAGGGCACGUGC
MIR164	Csa3M234520.1		UGGAGAAGCAGGGCACGUGC
MIR166	Csa7M452940.1	*Cucumis sativus* homeobox-leucine zipper protein ATHB-15-like (LOC101213106)	UCGGACCAGGCUUCAUUCCC
MIR166	Csa6M141360.1		UCGGACCAGGCUUCAUUCCC
MIR166	Csa1M538230.1		UCGGACCAGGCUUCAUUCCC
MIR166	Csa6M525430.1	*Cucumis sativus* homeobox-leucine zipper protein ATHB-9-like (LOC101210154)	UCGGACCAGGCUUCAUUCCC
MIR166	Csa6M150540.1		UCGGACCAGGCUUCAUUCCC
MIR166	Csa5M576830.2		UCGGACCAGGCUUCAUUCCC
MIR166	Csa5M576830.1	*Cucumis sativus* mitochondrial substrate carrier family protein W-like (LOC101223274)	UCGGACCAGGCUUCAUUCCC
MIR166	Csa3M776860.2		UCGGACCAGGCUUCAUUCCC
MIR166	Csa3M776860.3		UCGGACCAGGCUUCAUUCCC
MIR166	Csa3M776860.1	*Cucumis sativus* protein ABSCISIC ACID-INSENSITIVE 5-like (LOC101209887)	UCGGACCAGGCUUCAUUCCC
MIR167	Csa3M145240.1	NO BLAST	UGAAGCUGCCAGCAUGAUCU
MIR167	Csa1M702010.1		UGAAGCUGCCAGCAUGAUCUG
MIR167	Csa1M554540.1	*Cucumis sativus* UDP-arabinose 4-epimerase 1-like (LOC101214131), mRNA	UGAAGCUGCCAGCAUGAUCU
MIR169	Csa6M087830.1	*Cucumis sativus* UV-stimulated scaffold protein A homolog (LOC101209611)	AAGCCAAGGAUGAAUUGCCGG
MIR169	Csa1M038890.1	*Cucumis sativus* uncharacterized LOC101220892 (LOC101220892)	AAGCCAAGGAUGAAUUGCCGG
MIR172	Csa4M292470.1	*Cucumis sativus* ethylene-responsive transcription factor	GGAAUCUUGAUGAUGCUGCA,

Table 3.2 Possible miRNA targets in *C. maxima* (Continued).

MIR172	Csa2M279250.1	*Cucumis sativus* floral homeotic protein APETALA 2-like (LOC101208665)	GGAAUCUUGAUGAUGCUGCA
MIR172	Csa3M736760.1	*Cucumis sativus* ethylene-responsive transcription factor RAP2-7-like (LOC101211242)	GGAAUCUUGAUGAUGCUGCA
MIR172	Csa5M175970.1		GGAAUCUUGAUGAUGCUGCA
MIR172	Csa6M296960.2		GGAAUCUUGAUGAUGCUGCA
MIR172	Csa6M296960.1	*Cucumis sativus* ethylene-responsive transcription factor RAP2-7-like (LOC101226008)	GGAAUCUUGAUGAUGCUGCA
MIR172	Csa6M491020.1		GGAAUCUUGAUGAUGCUGCA
MIR390	Csa4M166920.1	*Cucumis sativus* leucine-rich repeat receptor-like tyrosine-protein kinase At2g41820-like	AAGCUCAGGAGGGAUAGCGCC
MIR390	Csa2M354040.1	*Cucumis sativus* uncharacterized LOC101212317 (LOC101212317)	AAGCUCAGGAGGGAUAGCGC
MIR390	Csa5M175820.1	*Cucumis sativus* cytokinin dehydrogenase 6-like (LOC101223981)	AAGCUCAGGAGGGAUAGCGC
MIR393	Csa3M597350.1	*Cucumis sativus* protein AUXIN SIGNALING F-BOX 2-like (LOC101222179)	UCCAAAGGGAUCGCAUUGAUCC
MIR393	Csa7M393970.1	*Cucumis sativus* protein TRANSPORT INHIBITOR RESPONSE 1-like (LOC101216544)	UCCAAAGGGAUCGCAUUGAU
MIR393	CsaUNM033720.1		UCCAAAGGGAUCGCAUUGAUC
MIR397	Csa6M445190.1	*Cucumis sativus* laccase-11-like (LOC101216806)	UCAUUGAGUGCAGCGUUGAUG
MIR397	Csa4M308480.2		UCAUUGAGUGCAGCGUUGAU
MIR397	Csa4M308480.1		UCAUUGAGUGCAGCGUUGAU
MIR397	Csa1M528530.1		UCAUUGAGUGCAGCGUUGAUG
MIR397	Csa3M732490.1		UCAUUGAGUGCAGCGUUGAU
MIR397	Csa3M734150.1	*Cucumis sativus* laccase-17-like (LOC101205653)	UCAUUGAGUGCAGCGUUGAU
MIR397	Csa4M004810.1	*Cucumis sativus* PWP2 periodic tryptophan protein homolog (yeast) (PWP2)	UCAUUGAGUGCAGCGUUGAU
MIR397	Csa3M734120.1		UCAUUGAGUGCAGCGUUGAU
MIR397	Csa7M394000.1		UCAUUGAGUGCAGCGUUGAUG
MIR397	Csa3M733340.1	*Cucumis sativus* BEL1-like homeodomain protein 9-like (LOC101221128)	UCAUUGAGUGCAGCGUUGAU

CHAPTER 4

DISCUSSION

4.1 TRANSCRIPTOME-WIDE MYB IDENTIFICATION IN BARLEY

4.1.1 Characterization of the barley R2R3-type MYB TFs

In this section of thesis, 51 R2R3-type MYB proteins were isolated from the barley RNA-seq data. Although the MYB genes comprise one of the largest TF families in plants (Pabo and Sauer, 1992; Riechmann et al., 2000), R2R3 MYB transcription factors are the most common type among plant MYBs. At the same time, R2R3 MYB gene numbers shows variety among the species. For instance, *A. thaliana* included 126 (Stracke et al. 2001; Yanhui et al. 2006; Dubos et al. 2010), *Populus trichocarpa* 192 (Wilkins et al. 2009), *Vitis vinifera* 108 (Matus et al. 2008), rice 88 (Katiyar et al. 2012) and wheat 22 (Zhang et al. 2012). In addition, soybean genome is harboring the largest R2R3 MYB family members (244 proteins) among the known plants (Du et al. 2012b). In our study, we demonstrated 51 R2R3 MYB proteins in the full transcriptome data of barley. The abundance of MYB genes in a species may be related with genome duplications (segmental/tandem), rather than the genome size. Wheat genome (17 Gb) include only 22 R2R3 MYB proteins (Zhang et al. 2012). Also, it is interesting that in some known species belonging to eudicot lineage (*A. thaliana*, *P. trichocarpa*, *V. vinifera*, *G. max*), the number of R2R3 MYB subfamily members was found over than that of known monocots (*O. sativa*, *T. aestivum*, *H. vulgare*) (Table 4.1). However, it should be considered that the total number of family members can be altered by the type of data source used in the study. Studies using the whole genome sequences might be useful to obtain precise protein abundances than the transcriptome-wide or EST-based data sources.

Table 4.1 Number of R2R3 MYB genes among known species with their chromosomal number, genomes size and lineage information.

Species	**# of R2R3 MYB**	**Clade**	**Chr. No (n)**	**Genome size**	**Reference**
G. max	244	Eudicots	20	1.1 Gb	Du et al. 2012b
P. trichocarpa	192	Eudicots	19	485 Mb	Wilkins et al. 2009
A. thaliana	126	Eudicots	5	157 Mb	Stracke et al. 2001; Yanhui et al. 2006; Dubos et al. 2010
V. vinifera	108	Eudicots	19	487 Mb	Matus et al. 2008
Z. mays	157	Monocots	10	2.5 Gb	Du et al. 2012a
O. sativa	88	Monocots	12	383 Mb	Katiyar et al. 2012
H. vulgare	51	Monocots	7	5 Gb	Current study
T. aestivum	22	Monocots	7	17 Gb	Zhang et al. 2012

4.1.2 Comparative analysis of R2R3 MYB DNA Binding Domain (DBD)

The main characteristics of MYB protein is three regular Trp (W) residues locating along with each DBD (6, 26, and 46 for the R2 repeat and 59, 78 and 97 for the R3). In order to clarify the barley MYB sequences whether they include conserved Trp residues in the right position; we compared the sequences with some known species (*Arabidopsis*, *Brachypodium*, maize), separately (Figure 3.2). Consistent with MYB family protein models as described for *Arabidopsis*, maize and soybean (Dubos et al. 2010; Du et al. 2012a; Du et al. 2012b), primary structure of DBD for barley was formed as follow: –W-(X_{19})-W-(X_{19})-W- …. –F/I-(X_{18})-W-(X_{18})-W-. 18 and 19 amino acids were found between the two Trp (W) residues of R2 and R3 repeats, respectively. Trp6, Trp46 and Trp78 residues were found systematically dispersed in all HvMYB proteins. Trp26 and Trp97 were highly conserved (~98%), except one MYB protein (HvMYB48) including an amino acid shift at position 24. In here, Trp (W) residue was located at 27 instead of 26. At the position 97, substitution was occurred with Phe (F) and Arg (R) residues for HvMYB48 and HvMYB51, respectively. Except Phe (F), others do not have hydrophobic side chain and not exactly complete the MYB DBD model. That may gain the protein new functions or can do them ineffective. Trp59 was found only 5 (~10%) among all R2R3 HvMYB proteins and it was the most differing

one among the conserved tryptophan residues in MYB repeats. At this position, Trp (W) mostly exchanged with non-polar and hydrophobic characterized Phe (F) (~70%). Met (M) (~12%), Leu (L) (~6%) and Ile (I) (~4%) were the other substituted residues that they have hydrophobic side chains, too. Most probably these exchanges do not affect the hydrophobic core structure of the DBD of MYBs due to their similar hydrophobic characteristics. Beside this, changing could gain different roles on divergent cellular processes in the cell. One another highly conserved residue is Cys42 (C) which was located at the third helix of DBD and determined at the same position of all R2R3 type MYB proteins.

As it was stated in a recent study published by Du et al. (2012a), LRPD motif composes the linker part of R2 and R3 repeats and was found to be highly conserved between *Arabidopsis* and barley. In the case of barley, we found that LRPD motif located between the positions Leu50 to Asn53 (Figure 3.1; Figure 3.2). However, in the animal models MYB counterpart myc had LNPE, instead of LRPD (Hegvold and Gabrielsen 1996). It was observed that barley MYB TF proteins were also included the consensus four-based linker part as in the case of other selected plant species (*Arabidopsis*, *Brachypodium*, maize), too (Figure 3.2 A-D). In common, unlike the animal MYBs, plant MYBs naturally has an additional Leu (L) insertion between the second and the third helices of R2 repeat. It was reported that Leu (L) was not present in the animal models. *In vitro* study on v-MYB protein revealed that insertion of Leu (L) residue between the second and the third helices has altered the DNA binding capacity of proteins (Williams and Grotewold 1997). In maize, 85% of all R2R3-MYB proteins included Leu (L) insertion (Du et al. 2012a). In our study, we observed that Leu35 inserted in almost 92% of all R2R3 MYB proteins of barley (Figure 3.1). With its equivalences, the other plant species also had the same insertion as in the case of barley (Figure 3.2). We conclude that plant MYBs may position the Leu (L) residue naturally. And as it was stated by Williams and Grotewold (1997), this may increase the DNA binding capacity positively. Another idea for insertion is that it could be a subsequent replacement with time due to MYB's possibly critical functions in plants. In addition, two of candidate proteins for MYB TFs (Unigene29223 and CL17477) were included unique 27 amino acid insertions which located between the first and the second Trp (W) residues in the R3 repeat (data not shown). Predicting the inserted portion by using WoLFPSORT program (Horton et al. 2007) gave no significant result. As we know, no attempt has been made previously regarding to their possible functions.

4.1.3 Comparative phylogenetic analysis of R2R3 MYB family proteins in barley and *Arabidopsis*

Protein structures and biological functions of the proteins residing into the same subgroups are correlated (Dubos et al. 2010). To predict the biological functions of barley MYBs, phylogenetic distances were computed comparatively with the *Arabidopsis* MYB TFs (Figure 3.6). For this aim, the generated subgroups were collectively added to that of barley R2R3 MYBs. By referring to the *Arabidopsis* functional categorization (Dubos et al. 2010), 15 of 51 barley MYB TFs were found at the same subgroup with *Arabidopsis*. They were categorized as defense, development and metabolism: HvMYB12, HvMYB23, HvMYB32, HvMYB33 with defense; HvMYB12, HvMYB29, HvMYB30, HvMYB32, HvMYB33, HvMYB41, HvMYB42 with development; and HvMYB16, HvMYB19, HvMYB20, HvMYB25, HvMYB26, HvMYB41, HvMYB42 with metabolism (Table 3.1). When the lack of sufficient data, phylogenetic distance based comparisons are useful for predicting the functions of putative proteins. In recent years, *Arabidopsis* MYB TFs have been well studied (Stracke et al. 2001; Dubos et al. 2010). *Arabidopsis* MYB TFs were used to predict the functional annotations of identified barley MYB TFs. Beside this, COG (Clusters of Orthologous Groups) categories to classify the MYB TFs were obtained from the RNA-Seq data of barley. Based on the two different functional classification methods, 51 R2R3 MYB TFs were assigned into the appropriate subgroups.

According to their functions, *Arabidopsis* R2R3 MYB proteins have been divided into 25 subgroups (Stracke et al. 2001; Dubos et al. 2010). In the present study, the number of subgroups was expanded to 34 (S1 to S34) (Figure 3.6). In the Figure 3.5, it was shown that HvMYB3, HvMYB4, HvMYB5, HvMYB6 and HvMYB15 were grouped into the same taxon and they were grouped under “cell cycle control, cell division, chromosome partitioning” class. As it was stated in Figure 3.3, except the HvMYB15, chromosomal locations of these genes were found at the same position in Chr6. Functional categorizations and chromosomal distribution of the MYB proteins revealed that these genes could be paraloguos and were in use for the same function in barley. Most notably, the case was appeared for HvMYB48, HvMYB49 and HvMYB51 proteins, too. As they were stated inside the colored frame (Figure 3.5), the three proteins were involved in “RNA processing and modification” class and fall into the

same taxon. Based on the chromosomal distribution, their locations were predicted in different chromosomes (Chr2, Chr1 and Chr5, respectively). Here, the three genes with high frequency sequence similarity could be paraloguos and may function in the regulation of cellular homeostasis, related with RNA modification processes during B-stress. To make their functions precise further experiments are needed.

4.1.4 Expression analysis of identified R2R3 MYB TFs in organs under Boron stress

When compared the MYB expressions between the non-stressed root and leaf samples (50R/50L), only three MYB transcripts expressions were found to be higher in the leaf samples (HvMYB20, HvMYB32 and HvMYB33). Of these, HvMYB32 and HvMYB33 were grouped in the same cluster (S1) in the phylogenetic tree (Figure 3.5; Figure 3.6) and showed high homology with AtMYB30, AtMYB31, AtMYB60 and AtMYB94. In the previous studies, it was found that AtMYB30 and AtMYB60 have role in the regulation of abiotic and biotic stresses, respectively (Cominelli et al. 2005; Raffaele et al. 2008). Remarkably, under B stress HvMYB15 and HvMYB37 were found highly repressed in roots. Beside this, both of them clustered in distinct groups (S27 and S30, respectively) from the *Arabidopsis* MYBs (Figure 3.6). In addition, in root samples the expressions were remarkably increased for HvMYB13, HvMYB40, HvMYB42 and HvMYB50. In the leaves, HvMYB40 was highly repressed; as well as HvMYB1, HvMYB11, HvMYB19, HvMYB28 and HvMYB49 were highly induced (Figure 3.7; Figure 4.1).

	50R/1000R	50L/1000L	50R/50L	1000R/1000L
HvMYB01	1,33	0,04	207,00	6,28
HvMYB02	0,00	0,00	0,00	0,00
HvMYB03	0,00	0,00	0,00	0,00
HvMYB04	0,00	0,00	0,00	0,00
HvMYB05	0,00	0,00	0,00	0,00
HvMYB06	0,00	0,00	0,00	0,00
HvMYB07	1,09	2,46	76,92	190,00
HvMYB08	0,00	0,00	0,00	0,00
HvMYB09	3,45	0,48	385,00	76,00
HvMYB10	1,33	0,40	21,25	7,00
HvMYB11	4,53	0,31	438,00	29,40
HvMYB12	0,76	0,00	115,38	40,00
HvMYB13	0,45	1,36	76,47	280,00
HvMYB14	0,60	2,86	3,95	19,60
HvMYB15	38,00	0,00	100,00	80,00
HvMYB16	0,67	0,66	3,63	3,20
HvMYB17	2,60	0,00	0,00	1,10
HvMYB18	0,88	1,10	111,50	135,00
HvMYB19	1,20	0,42	313,30	122,00
HvMYB20	0,00	3,24	0,02	0,01
HvMYB21	1,26	0,52	40,00	18,00
HvMYB22	1,45	0,46	3,60	1,14
HvMYB23	0,91	3,23	80,95	98,70
HvMYB24	0,75	0,00	32,90	44,20
HvMYB25	1,36	1,70	123,17	156,42
HvMYB26	1,52	3,50	80,00	18,50
HvMYB27	1,62	0,42	194,44	52,50
HvMYB28	1,22	0,32	241,11	81,20
HvMYB29	0,84	0,00	29,00	35,50
HvMYB30	1,03	0,00	195,30	189,01
HvMYB31	1,10	0,36	7,81	2,80
HvMYB32	2,00	0,02	0,30	0,01
HvMYB33	0,00	0,65	0,01	0,01
HvMYB34	0,00	0,00	0,00	14,10
HvMYB35	0,53	3,00	327,77	111,31
HvMYB36	0,67	0,00	52,00	86,45
HvMYB37	18,00	0,00	214,55	9,02
HvMYB38	1,10	0,00	3,00	27,00
HvMYB39	1,54	0,00	67,00	43,69
HvMYB40	0,23	71,00	0,00	3,40
HvMYB41	3,22	0,00	52,11	14,95
HvMYB42	0,46	1,14	56,66	149,20
HvMYB43	1,41	1,50	5,83	6,22
HvMYB44	1,18	0,65	4,43	2,45
HvMYB45	1,12	0,81	25,02	18,75
HvMYB46	0,73	0,88	38,00	78,20
HvMYB47	1,93	0,00	315,00	163,62
HvMYB48	0,94	1,01	2,98	3,21
HvMYB49	0,80	0,05	46,00	2,43
HvMYB50	0,44	0,64	2,50	3,75
HvMYB51	0,94	0,62	3,05	2,01

Figure 4.1 Comparative expression analysis of 51 MYB TFs under B stress.

To verify the expressions, six MYB TF genes (HvMYB51, HvMYB09, HvMYB14, HvMYB35, HvMYB47 and HvMYB45) were randomly selected and quantitatively analyzed by using RT-qPCR. Quantitative results were normalized by using *18S rRNA* (AY552749.1) barley transcripts (Figure 4.2). The results showed that RNA-seq data is consistent with qPCR results. The relative expression changes of the selected genes were similar with RPKM ratios. It revealed that the data obtained for expression analysis are reliable.

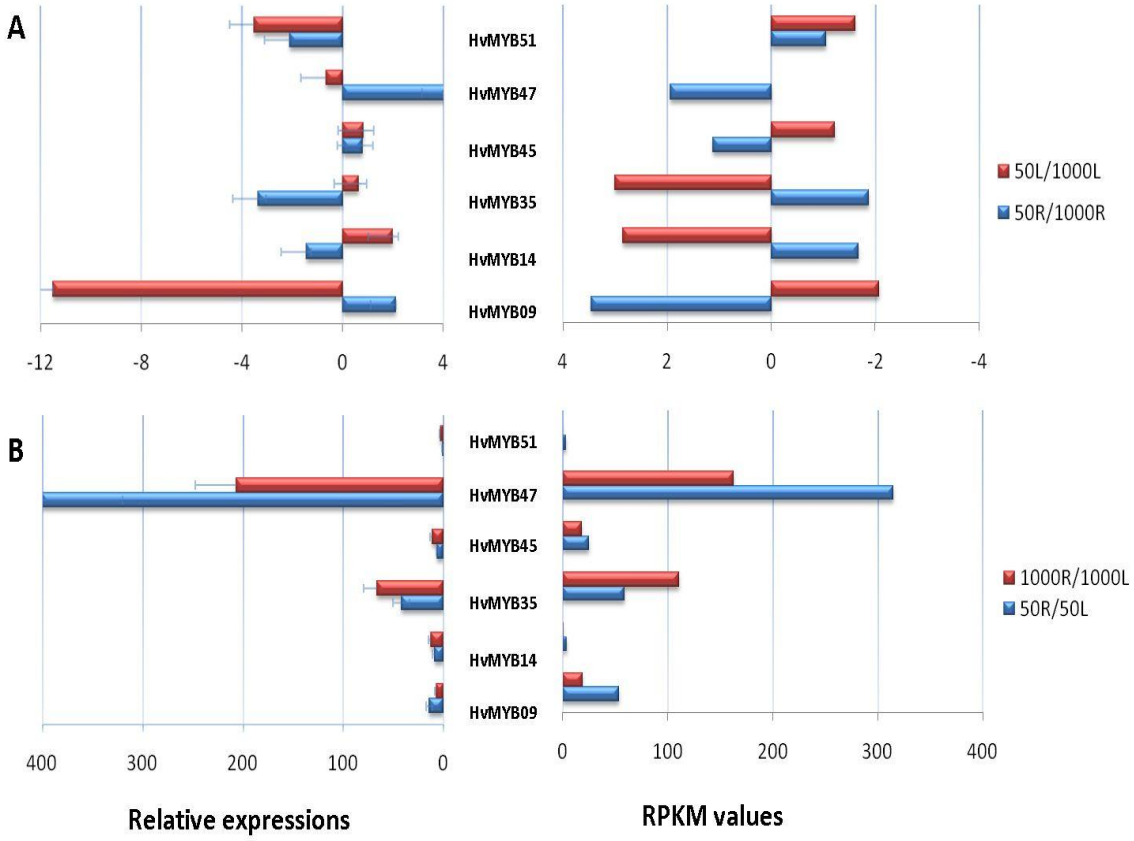

Figure 4.2 Expression analyses of six selected MYB TFs with their RPKM ratio**.** Stressed and control root and leaf samples of the genes were quantified by RT-qPCR and normalized with *18S rRNA* transcripts. Transcript accumulations were assessed through 50L/1000L and 50R/1000R (control and B-stressed) (A) and 50R/50L and 1000R/1000L (B) samples. The results were compared with RNA-seq expression data (RPKM). Error bars represent the standard errors of the means calculated from three Cp values of two independent experiments. 50R and 50L represent control root and leaf; 1000R and 1000L represent Boron stressed root and leaf sample (1000 µM), respectively.

Table 4.2 HvMYB proteins submitted to NCBI database. The following BioSample IDs and URLs were given for those proteins.

Sample Name	**Tax ID**	**BioProject ID**	**Object IDs and corresponding URLs:**
HvMYB01	4513	188758	1915685: http://www.ncbi.nlm.nih.gov/biosample/1915685
HvMYB02	4513	188758	1915686: http://www.ncbi.nlm.nih.gov/biosample/1915686
HvMYB03	4513	188758	1915687: http://www.ncbi.nlm.nih.gov/biosample/1915687
HvMYB04	4513	188758	1915688: http://www.ncbi.nlm.nih.gov/biosample/1915688
HvMYB05	4513	188758	1915689: http://www.ncbi.nlm.nih.gov/biosample/1915689
HvMYB06	4513	188758	1915690: http://www.ncbi.nlm.nih.gov/biosample/1915690
HvMYB07	4513	188758	1915691: http://www.ncbi.nlm.nih.gov/biosample/1915691
HvMYB08	4513	188758	1915692: http://www.ncbi.nlm.nih.gov/biosample/1915692
HvMYB09	4513	188758	1915693: http://www.ncbi.nlm.nih.gov/biosample/1915693
HvMYB10	4513	188758	1915694: http://www.ncbi.nlm.nih.gov/biosample/1915694
HvMYB11	4513	188758	1915695: http://www.ncbi.nlm.nih.gov/biosample/1915695
HvMYB12	4513	188758	1915696: http://www.ncbi.nlm.nih.gov/biosample/1915696
HvMYB13	4513	188758	1915697: http://www.ncbi.nlm.nih.gov/biosample/1915697
HvMYB14	4513	188758	1915698: http://www.ncbi.nlm.nih.gov/biosample/1915698
HvMYB15	4513	188758	1915699: http://www.ncbi.nlm.nih.gov/biosample/1915699
HvMYB16	4513	188758	1915700: http://www.ncbi.nlm.nih.gov/biosample/1915700
HvMYB17	4513	188758	1915701: http://www.ncbi.nlm.nih.gov/biosample/1915701
HvMYB18	4513	188758	1915702: http://www.ncbi.nlm.nih.gov/biosample/1915702
HvMYB19	4513	188758	1915703: http://www.ncbi.nlm.nih.gov/biosample/1915703
HvMYB20	4513	188758	1915704: http://www.ncbi.nlm.nih.gov/biosample/1915704
HvMYB21	4513	188758	1915705: http://www.ncbi.nlm.nih.gov/biosample/1915705
HvMYB22	4513	188758	1915706: http://www.ncbi.nlm.nih.gov/biosample/1915706
HvMYB23	4513	188758	1915707: http://www.ncbi.nlm.nih.gov/biosample/1915707
HvMYB24	4513	188758	1915708: http://www.ncbi.nlm.nih.gov/biosample/1915708
HvMYB25	4513	188758	1915709: http://www.ncbi.nlm.nih.gov/biosample/1915709
HvMYB26	4513	188758	1915710: http://www.ncbi.nlm.nih.gov/biosample/1915710
HvMYB27	4513	188758	1915711: http://www.ncbi.nlm.nih.gov/biosample/1915711
HvMYB28	4513	188758	1915712: http://www.ncbi.nlm.nih.gov/biosample/1915712
HvMYB29	4513	188758	1915713: http://www.ncbi.nlm.nih.gov/biosample/1915713
HvMYB30	4513	188758	1915714: http://www.ncbi.nlm.nih.gov/biosample/1915714
HvMYB31	4513	188758	1915715: http://www.ncbi.nlm.nih.gov/biosample/1915715

Table 4.2 HvMYB proteins submitted to NCBI database. The following BioSample IDs and URLs were given for those proteins (Continued).

HvMYB32	4513	188758	1915716: http://www.ncbi.nlm.nih.gov/biosample/1915716
HvMYB33	4513	188758	1915717: http://www.ncbi.nlm.nih.gov/biosample/1915717
HvMYB34	4513	188758	1915718: http://www.ncbi.nlm.nih.gov/biosample/1915718
HvMYB35	4513	188758	1915719: http://www.ncbi.nlm.nih.gov/biosample/1915719
HvMYB36	4513	188758	1915720: http://www.ncbi.nlm.nih.gov/biosample/1915720
HvMYB37	4513	188758	1915721: http://www.ncbi.nlm.nih.gov/biosample/1915721
HvMYB38	4513	188758	1915722: http://www.ncbi.nlm.nih.gov/biosample/1915722
HvMYB39	4513	188758	1915723: http://www.ncbi.nlm.nih.gov/biosample/1915723
HvMYB40	4513	188758	1915724: http://www.ncbi.nlm.nih.gov/biosample/1915724
HvMYB41	4513	188758	1915725: http://www.ncbi.nlm.nih.gov/biosample/1915725
HvMYB42	4513	188758	1915726: http://www.ncbi.nlm.nih.gov/biosample/1915726
HvMYB43	4513	188758	1915727: http://www.ncbi.nlm.nih.gov/biosample/1915727
HvMYB44	4513	188758	1915728: http://www.ncbi.nlm.nih.gov/biosample/1915728
HvMYB45	4513	188758	1915729: http://www.ncbi.nlm.nih.gov/biosample/1915729
HvMYB46	4513	188758	1915730: http://www.ncbi.nlm.nih.gov/biosample/1915730
HvMYB47	4513	188758	1915731: http://www.ncbi.nlm.nih.gov/biosample/1915731
HvMYB48	4513	188758	1915732: http://www.ncbi.nlm.nih.gov/biosample/1915732
HvMYB49	4513	188758	1915733: http://www.ncbi.nlm.nih.gov/biosample/1915733
HvMYB50	4513	188758	1915734: http://www.ncbi.nlm.nih.gov/biosample/1915734
HvMYB51	4513	188758	1915735: http://www.ncbi.nlm.nih.gov/biosample/1915735

4.2 QUANTITATIVE ANALYSIS OF PHLOEM-DERIVED Cuc-miRNAs UNDER BORON STRESS

In this section of the thesis, the responses of 14 phloem-derived *C. maxima* miRNAs were evaluated against B-stress induction. Phloem saps (PS) were collected from the control and B-induced plants and PS RNA isolations were carried out. Stem-loop RT-qPCR method was used to analyze the miRNA expression. This method is very useful and efficient method which has been firstly proposed by Chen et al. (2005). The method allows precise quantification with little amount of 25 pg of total RNA for most miRNAs (Chen et al., 2005).

Phloem-derived miRNAs have been discovered from various plants including *Brassica napus* (rapeseed) (Buhtz et al., 2008; Buhtz et al., 2010), *Cucurbita maxima* (pumpkin) (Buhtz et al., 2010), *Malus domestica* (apple) (Varkonyi-Gasic et al., 2010), and *Lupinus albus* (white lupin) (Atkins and Smith, 2007). In the phloem sap of those plants, 24 different miRNAs were detected by using several methods such as qPCR, sequencing, RNA gel blotting and array techniques (Kehr, 2012). In these studies some miRNAs have been observed as stress responsive and/or phloem-mobile. For instance, miR169 was defined as responsive under conditions of excess phosphate and nitrogen (Pant et al., 2009). Also, miR158 was found to be over-expressed in iron deficiency (Buhtz et al., 2010). In cupper deficient conditions, miR397, miR398, miR399, miR408 and miR2111 were detected as responsive (Buhtz et al., 2008). In addition miR172 and miR399 were defined as phloem-mobile molecules that they were acted as a signal molecule during the stress response over long distance communication.

In here, 14 miRNAs were selected as a candidate long distance signal responsive miRNAs in pumpkin. All are detected in the phloem sap of either in control and B-subjected plant phloem sap exudates. Some of those miRNA molecules were found to be over-expressed against B-induction. We propose that those miRNAs may be correlated with B stress and acts as a B-responsive signal molecule. For instance in the quantitation analysis, it was found that the accumulations of Cma-miR156, Cma-miR160, Cma-miR166, Cma-miR169, Cma-miR172, and Cma-miR393 were increased against B-induction. When we compared the relative expressions, some miRNAs were found to be really over-expressed even some miRNA expressions were seem to be B-stress dependent. For instance, Cma-miR156, Cma-miR160, Cma-miR166, and Cma-miR172 were regularly resided in PS of control plants. However, Cma-miR169 and Cma-miR393 expressions were observed only in B-stress induced plants. This result indicated that Cma-miR156, Cma-miR160, Cma-miR166, and Cma-miR172 were slightly responded against B-stress. However, Cma-miR169 and Cma-miR393 were highly accumulated in the PS of pumpkin against B-stress. Further experiment can be focused on especially to prove the transport ability of miR169 and miR172, either conducting grafting experiments or Nothern blotting.

CHAPTER 5

CONCLUSION

In this dissertation, we identified 51 R2R3-type MYB TFs from the RNA-seq data of B-tolerant barley. Considering the phylogenetic analysis, they were divided into distinct functional categories and their putative functions were predicted. Also, their expression analysis was conducted between organs under B-stressed condition. Some MYBs were noted as important TFs during B regulatory processes. MYB TFs could be important regulators in tolerant/intolerant cultivars of barley. In Sahara, as being a B tolerant cultivar, MYB genes could be important players in B-tolerance mechanisms. Although some TFs have been found to be essential under low or high B conditions (Kasajima et al., 2010; Ochiai et al., 2011), no attempt has been made previously regarding the interaction with MYB TFs and B homeostasis. For the further functional analysis, the phylogenetic and expression data can help for the selection of candidate TFs that are possibly involved in the regulation of B-toxicity tolerance mechanism. Also, the data represented here may contribute to the understanding of gene functions for any other susceptible mechanisms in barley.

In addition, according to the results about the long distance communications of plants, we proposed six miRNAs (Cma-miR156, Cma-miR160, Cma-miR166, and Cma-miR172, Cma-miR169, and Cma-miR393) which they were found in the phloem sap of pumpkin and they were induced against B-stress. Especially miR169 and miR393 were very remarkable regarding their expression profiling. These miRNAs may possibly have role in the long distance signal transduction mechanism when the plants are induced by excessive B. Regarding to the possible mechanism of miRNA movement; we proposed that some proteins may serve as an escorting molecule over long distances. Another hypothesis could be the protein-RNA interaction. As we know, mobile RNAs

can interact with special proteins for translocation, especially over long distances. However, this phenomenon is not exactly right and clearly identified yet.

The question about the long distance travelling of miRNA is still a question mark. Are they travelling alone or they have a comrade escorting to them? In this dissertation, we revealed some unique miRNAs responded against B-stress. Are the plants sending stress signals to their aerial parts to protect them against the stresses? If yes, can those miRNA molecules act as signal molecules? By means of our results, further experiments can be conducted to find these answers.

REFERENCES

Abe, H., T. Urao, et al., ″*Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling.″ *Plant Cell*, Vol. 15, pp. 63–78, 2003.

Agarwal, M., Y. Hao, et al., ″A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance.″ *J Biol Chem*, Vol. 281, pp. 37636–37645, 2006.

Apweiler, R., T. K. Attwood, et al., ″The InterPro database, an integrated documentation resource for protein families, domains and functional sites.″ *Nucleic Acids Res*, Vol. 29, pp. 37–40, 2001.

Altschul, S. F., T. L. Madden, et al., ″Gapped BLAST and PSI-BLAST: A new generation of protein database search programs.″ *Nucleic Acids Res*, Vol. 25, pp. 3389–3402, 1997.

Atkins, C. A., and P. M. C. Smith, ″Translocation in legumes: assimilates, nutrients, and signaling molecules.″ *Plant Physiol*, Vol. 144, pp. 550–561, 2007.

Aung, K., S. L. Lin, et al., ″pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene.″ *Plant Physiol*, Vol. 141(3), pp. 1000-1011, 2006.

Bailey, T. L., N. Williams, et al., ″MEME: discovering and analyzing DNA and protein sequence motifs.″ *Nucleic Acids Res*, Vol. 34, pp. W369–W373, 2006.

Banerjee, A. K., M. Chatterjee, et al., ″Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway.″ *Plant Cell*, Vol. 18, pp. 3443–3457, 2006.

Banerjee, A. K., Lin, T., D. J. Hannapel, ″Untranslated regions of a mobile transcript mediate RNA metabolism.″ *Plant Physiol*, Vol. 151(4), pp. 1831-1843, 2009.

Bari, R., B. D. Pant, et al., ″PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants.″ *Plant Physiol*, Vol. 141, pp. 988-999, 2006.

Borsani, O., J. Zhu, et al., ″Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis.″ *Cell*, Vol. 123, pp. 1279–1291, 2005.

Buhtz, A., F. Springer, et al., ″Identification and characterization of small RNAs from the phloem of *Brassica napus*.″ *Plant J*, Vol. 53, pp. 739–749, 2008.

Buhtz, A., J. Pieritz, et al., ″Phloem small RNAs, nutrient stress responses, and systemic mobility.″ *BMC Plant Biol*, Vol. 10, pp. 64, 2010.

Burch-Smith, T. M., P. C. Zambryski, ″Plasmodesmata paradigm shift: regulation from without versus within.″ *Annu Rev Plant Biol*, Vol. 63, pp. 239-260, 2012.

Camacho-Cristobal, J. J., J. Rexach, et al., ″Boron in plants: deficiency and toxicity.″ *J Integr Plant Biol*, Vol. 50: pp. 1247–1255, 2008.

Chen, X., ″MicroRNA biogenesis and function in plants.″ *FEBS letters*, Vol. 579(26), pp. 5923-5931, 2005.

Chen, C., Ridzon, D. A., A. J. Broomer, et al., ″Real-time quantification of microRNAs by stem–loop RT–PCR.″ *Nucleic Acids Res*, Vol. 33, pp. e179-e179, 2005.

Chiou, T. J., ″The role of microRNAs in sensing nutrient stress.″ *Plant Cell Environ*, Vol. 30, pp. 323–332, 2007.

Chomczynski, P., and N. Sacchi, ″Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.″ *Anal Biochem*, Vol. 162, pp. 156-159, 1987.

Cominelli, E., M. Galbiati, et al., ″A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance.″ *Curr Biol*, Vol. 15, pp. 1196–1200, 2005.

Crooks, G.E., G. Hon, et al., "WebLogo: A sequence logo generator." *Genome Res*, Vol. 14, pp. 1188–1190, 2004.

Dai, X., Y. Xu, et al., ″Over-expression of an R1R2R3 MYB gene, Os*MYB3R-2*, increases tolerance to freezing, drought, and salt stress in transgenic *Arabidopsis*.″ *Plant Physiol*, Vol. 143, pp. 1739–1751, 2007.

Dai, X., Zhao, P. X., ″psRNATarget: a plant small RNA target analysis server.″ *Nucleic Acids Res*, Vol. 39, pp. W155-W159, 2011.

Dai, X., Y. Wang, et al., ″Os*MYB2P-1*, an R2R3 MYB transcription factor, is involved in the regulation of phosphate-starvation responses and root architecture in rice.″ *Plant Physiol*, Vol. 159, pp. 169–183, 2012.

Denekamp, M., and S. C. Smeekens, ″Integration of wounding and osmotic stress signals determines the expression of the AtMYB102 transcription factor gene.″ *Plant Physiol*, Vol. 132, pp. 1415–1423, 2003.

Ding, Z., S. Li, et al., ″Transgenic expression of *MYB15* confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*.″ *J Genet Genomics*, Vol. 36, pp. 17–29, 2009.

Doering-Saad, C., H. J. Newbury, ″A phloem-enriched cDNA library from Ricinus: insights into phloem function.″ *J Exp Bot*, Vol. 57, pp. 3183–3193, 2006.

Du, H., B. Feng, et al., ″The R2R3-MYB transcription factor gene family in maize.″ *PLoS ONE*, Vol. 7(6), pp. e37463, 2012a.

Du, H., S. Yang, et al., ″Genome-wide analysis of the MYB transcription factor superfamily in soybean.″ *BMC Plant Biol*, Vol. 12, pp. 106, 2012b.

Dubos, C., R. Stracke, et al., ″MYB transcription factors in *Arabidopsis*.″ *Trends Plant Sci*, Vol. 15, pp. 573–581, 2010.

Dunoyer, P., C. A. Brosnan, et al., ″An endogenous, systemic RNAi pathway in plants.″ *EMBO J*, Vol. 29(10), pp. 1699-1712, 2010.

Eldem, V., U. Ç. Akçay, et al., ″Genome-wide identification of miRNAs responsive to drought in peach (Prunus persica) by high-throughput deep sequencing.″ *PloS One*, Vol. 7(12), pp. e50298, 2012.

Fahlgren, N. et al., ″High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes.″ *PLoS ONE*, Vol. 2, pp. e219, 2007.

Feller, A., K. Machemer, et al., ″Evolutionary and comparative analysis of MYB and bHLH plant transcription factors.″ *Plant J*, Vol. 66, pp. 94–116, 2011.

Ganesan, G., H. M. Sankararamasubramanian, et al., ″A MYB transcription factor from the grey mangrove is induced by stress and confers NaCl tolerance in tobacco.″ *J Exp Bot*, Vol. 63(12), pp. 4549-4561, 2012.

Gao, N., Su, Y., Min, J., Shen, W., & Shi, W., ″Transgenic tomato overexpressing ath-miR399d has enhanced phosphorus accumulation through increased acid phosphatase and proton secretion as well as phosphate transporters.″ *Plant Soil*, Vol. 334, pp. 123-136, 2010.

Giavalisco, P., K. Kapitza, et al., ″Towards the proteome of *Brassica napus* phloem sap.″ *Proteomics*, Vol. 6, pp. 896–909, 2006.

Goodstein, D. M., S. Shu, et al., ″Phytozome: a comparative platform for green plant genomics.″ *Nucleic Acids Res*, Vol. 40, pp. D1178–D1186, 2012.

Gursanscky, N. R., and B. J. Carroll, ″Mechanism of Small RNA Movement″, in Kragler, F. and Hülskamp M. (Eds.), *Short and Long Distance Signaling*, pp. 99-131, Springer, New York, 2012.

Ham, B. K., J. L. Brandom, et al., ″A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex.″ *Plant Cell Online*, Vol. 21(1), pp. 197-215, 2009.

Hannapel, D. J., ″A perspective on photoperiodic phloem-mobile signals that control development.″ *Frontiers Plant Sci*, Vol. pp. 4, 2013.

Hannapel, D. J., Sharma, P., T. Lin, ″Phloem-mobile messenger RNAs and root development.″ *Frontiers Plant Sci*, Vol.1 pp. 4, 2013.

Harada, T., ″Grafting and RNA transport via phloem tissue in horticultural plants.″ *Scientia Horticulturae*, Vol. 125(4), pp. 545-550, 2010.

Haywood, V., T. Yu, et al., ″Phloem long-distance traffi cking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development.″ *Plant J*, Vol. 42, pp. 49–68, 2005.

Hegvold, A. B., and O. S. Gabrielsen, ″The importance of the linker connecting the repeats of the c-Myb oncoprotein may be due to a positioning function.″ *Nucleic Acids Res*, Vol. 24, pp. 3990–3995, 1996.

Hoagland, D. R., and D. I. Arnon, ″The water-culture method for growing plants without soil.″ *Circular. California Agri Exp Station*, 347 (2nd edit), 1950.

Horton, P., K. J. Park, et al., ″WoLF PSORT: protein localization redictor.″ *Nucleic Acids Res*, Vol. 35, pp. W585–W587, 2007.

Huang, N. C., T. S. Yu, ″The sequences of Arabidopsis GA-INSENSITIVE RNA constitute the motifs that are necessary and sufficient for RNA long-distance trafficking.″ *Plant J*, Vol. 59(6), pp. 921-929, 2009.

Jagadeeswaran, G., A. Saini, A, et al., ″Biotic and abiotic stress down-regulate miR398 expression in Arabidopsis.″ *Planta*, Vol. 229, pp. 1009–1014, 2009.

Jagadeeswaran, G., Nimmakayala, P., Y. Zheng, et al., ″Characterization of the small RNA component of leaves and fruits from four different cucurbit species.″ *BMC Genomics*, Vol. 13(1), pp. 329, 2012.

Jia, L., M. T. Clegg, et al., ″Evolutionary dynamics of the DNA-binding domains in putative R2R3-MYB genes identified from rice subspecies indica and japonica genomes.″ *Plant Physiol*, Vol. 134, pp. 575–585, 2004.

Jones-Rhoades, M. W., Bartel, D. P., B. Bartel, ″MicroRNAs and their regulatory roles in plants.″ *Annu Rev Plant Biol*, Vol. 57, pp. 19-53, 2006.

Juarez, M. T., Twigg, R. W., M. C. Timmermans, ″Specification of adaxial cell fate during maize leaf development.″ *Development*, Vol. 131(18), pp. 4533-4544, 2004.

Jung, C., J. S. Seo, et al., ″Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*.″ *Plant Physiol*, Vol. 146, pp. 623–635, 2008.

Kanehira, A., K. Yamada, et al., ″Apple phloem cells contain some mRNAs transported over long distances.″ *Tree Genetics & Genomes*, Vol. 6(5), pp. 635-642, 2010.

Kanei-Ishii, C., A. Sarai, et al., ″The tryptophan cluster: a hypothetic structure of the DNA-binding domain of the myb protooncogene product.″ *J Biol Chem*, Vol. 265, pp. 19990–19995, 1990.

Kantar, M., Unver, T., Budak, H., ″Regulation of barley miRNAs upon dehydration stress correlated with target gene expression.″ *Funct Integr Genomics*, Vol. 10, pp. 493–507, 2010.

Kasajima, I., Y. Ide, et al., ″WRKY6 is involved in the response to boron deficiency in *Arabidopsis thaliana*.″ *Physiol Plantarum*, Vol. 139, pp. 80–92, 2010.

Katiyar-Agarwal, S., S. Gao, et al., ″A novel class of bacteria-induced small RNAs in Arabidopsis.″ *Genes Development*, Vol. 21(23), pp. 3123-3134, 2007.

Katiyar, A., S. Smita, et al., ″Genome-wide classification and expression analysis of MYB transcription factor families in rice and *Arabidopsis*.″ *BMC Genomics*, Vol. 13, pp. 544, 2012.

Kehr, J. and A. Buhtz, ″Long distance transport and movement of RNA through the phloem.″ *J Exp Bot*, Vol. 59, pp. 85–92, 2008.

Kehr, J., ″Long-Distance Signaling by Small RNAs″, in Kragler, F. and Hülskamp M. (Eds.), *Short and Long Distance Signaling*, pp. 131-151, Springer, New York, 2012.

Kehr, J., ″Systemic regulation of mineral homeostasis by micro RNAs.″ *Frontiers Plant Sci*, Vol. pp. 4, 2013.

Kidner, C. A., R. A. Martienssen, ″Spatially restricted microRNA directs leaf polarity through ARGONAUTE1.″ *Nature*, Vol. 428, pp. 81-84, 2004.

Kim, M, W. Canio, et al., ″Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato.″ *Science*, Vol. 293, pp. 287–289, 2001.

Klempnauer, K., Gonda, T., Bishop, J., ″Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene.″ *Cell*, Vol. 31, pp. 453–463, 1982.

Kollmann, R., Dörr, I., Kleinig, H., ″Protein filaments-structural components of the phloem exudate.″ *Planta*, Vol. 95(1), pp. 86-94, 1970.

Kragler, F., ″RNA in the phloem: a crisis or a return on investment?″ *Plant Sci*, Vol. 178, pp. 99–104, 2010.

Kudo, H., Harada, T., ″A graft-transmissible RNA from tomato rootstock changes leaf morphology of potato scion. ″ *HortScience*, Vol. 42(2), pp. 225-226, 2007.

Kuhn, C., V. R. Franceschi, et al., ″Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements.″ *Science*, Vol. 275, pp. 1298–1300, 1997.

Latchman, D. S., ″Transcription factors: an overview.″ *Int J Biochem Cell B*, Vol. 29, pp. 1305–1312, 1997.

Lee, J. Y., J. Zhou, ″Function and identification of mobile transcription factors.″ In *Short and Long Distance Signaling*, pp. 61-86. Springer New York, 2012.

Letunic, I., Doerks, T., Bork, P., ″SMART7: recent updates to the protein domain annotation resource.″ *Nucleic Acids Res*, Vol. 40, pp. 302–305, 2012.

Li, W. X., Y. Oono, et al., ″The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance.″ *Plant Cell Online*, Vol. 20(8), pp. 2238-2251, 2008.

Li, B., Y. Qin, et al., ″Genome-wide characterization of new and drought stress responsive microRNAs in Populus euphratica.″ *J Exp Bot*, Vol. 62, pp. 3765-3779, 2011.

Liang, Y. K., C. Dubos, et al., ″AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*.″ *Curr Biol*, Vol. 15, pp. 1201–1206, 2005.

Lin, S. I., S. F. Chiang, et al., ″Regulatory network of microRNA399 and PHO2 by systemic signaling.″ *Plant Physiol*, Vol. 147(2), pp. 732-746, 2008.

Lin, T., P. Sharma, et al., ″The Impact of the Long-Distance Transport of a BEL1-Like Messenger RNA on Development.″ *Plant Physiol*, Vol. 161(2), pp. 760-772, 2013.

Lippold, F., D. H. Sanchez, et al., ″AtMyb41 regulates transcriptional and metabolic responses to osmotic stress in *Arabidopsis*.″ *Plant Physiol*, Vol. 149, pp. 1761–1772, 2009.

Lipsick, J. S., ″One billion years of Myb.″ *Oncogene*, Vol. 13, pp. 223–235, 1996.

Matus, J. T., F. Aquea, et al., ″Analysis of the grapeMYB R2R3 subfamily reveals expanded wine quality-related clades and conserved gene structure organization across *Vitis* and *Arabidopsis* genomes.″ *BMC Plant Biol*, Vol. 8, pp. 83, 2008.

Mayer, K. F., M. Martis, et al., ″Unlocking the barley genome by chromosomal and comparative genomics.″ *Plant Cell*, Vol. 23, pp. 1249-1263, 2011.

Moldovan, D., A. Spriggs, et al., ″Hypoxia-responsive microRNAs and trans -acting small interfering RNAs in Arabidopsis.″ *J Exp Bot*, Vol. 61, pp. 165–177, 2010.

Molnar, A., C. W. Melnyk, et al., ″Small silencing RNAs in plants are mobile and direct epigenetic modifi cation in recipient cells.″ *Science*, Vol. 328, pp. 872–875, 2010.

Mortazavi, A., B. A. Williams, et al., ″Mapping and quantifying mammalian transcriptomes by rna-seq.″ *Nat Methods*, Vol. 5, pp. 621-628, 2008.

Navarro, L., P. Dunoyer, et al., ″A plant miRNA contributes to antibacterial resistance by repressing auxin signaling.″ *Science,* Vol. 312, pp. 436-439, 2006.

Nozawa, A., K. Miwa, et al., ″Isolation of *Arabidopsis thaliana* cDNAs that confer yeast boric acid tolerance.″ *Biosci Biotech Bioch,* Vol. 70, pp. 1724–1730, 2006.

Ochiai, K., A. Shimizu, et al., ″Suppression of a NAC-like transcription factor gene improves boron-toxicity tolerance in rice.″ *Plant Physiol,* Vol. 156, pp. 1457–1463, 2011.

Ogata, K., C. Kanei-Ishii, et al., ″The cavity in the hydrophobic core of Myb DNA-binding domain is reserved for DNA recognition and trans-activation.″ *Nat Struct Biol,* Vol. 3, pp. 178–187, 1996.

Omid, A., Keilin, T., Glass, A., Leshkowitz, D., S. Wolf, ″Characterization of phloem-sap transcription profile in melon plants.″ *J Exp Bot,* Vol. 58(13), pp. 3645-3656, 2007.

Oparka, K. J., R. Turgeon, Sieve elements and companion cells—traffic control centers of the phloem. *Plant Cell Online,* Vol. 11(4), pp. 739-750, 1999.

Pabo, C. O., and R. T. Sauer, ″Transcription factors: structural families and principles of DNA recognition.″ *Annu Rev Biochem,* Vol. 6, pp. 1053–1095, 1992.

Palauqui, J., T. Elmayan, et al., ″Systemic acquired silencing: transgene-specifi c post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions.″ *EMBO J,* Vol. 16, pp. 4738–4745, 1997.

Pant, B. D., Buhtz, A., Kehr, J., Scheible, W. R., ″MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis.″ *Plant J,* Vol. 53(5), pp. 731-738, 2008.

Pant, B. D., M. Musialak-Lange, et al., ″Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing.″ *Plant Physiol,* Vol. 150(3), pp. 1541-1555, 2009.

Pasquali, G., S. Biricolti, et al., ″Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples.″ *Plant Cell Rep,* Vol. 27, pp. 1677–1686, 2008.

Paz-Ares, J., D. Ghosal, et al., ″The regulatory c1 locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators.″ *EMBO J,* Vol. 6, pp. 3553–3558, 1987.

Pfaffl, M. W., ″A new mathematical model for relative quantification in realtime RT-PCR.″ *Nucleic Acids Res,* Vol. 29(9), pp. 2002–2007, 2001.

Raffaele, S., F. Vailleau, et al., ″A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*.″ *Plant Cell*, Vol. 20, pp. 752–767, 2008.

Reid, R., ″Update on boron toxicity and tolerance in plants″, in Xu, F., Goldbach, H. E., Brown, P. H., Bell, R. W., Fujiwara, T., Hunt, C. D., Goldberg, S., Shi, L. (Eds.), *Advances in plant and animal boron nutrition*, pp. 83–90, Springer, Dordrecht, The Netherlands, 2007.

Riechmann, J. L., J. Heard, et al., ″*Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes.″ *Science*, Vol. 290, pp. 2105–2110, 2000.

Rozen, S., and H. J. Skaletsky, ″Primer3 on the WWW for General Users and for Biologist Programmers″, in Krawetz, S., Misener, S. (Eds.), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, pp. 365–386, Humana Press, Totowa, 2000.

Ruiz-Ferrer, V., O. Voinnet, ″Roles of plant small RNAs in biotic stress responses.″ *Annu Rev Plant Biol*, Vol. 60, pp. 485–510, 2009.

Ruiz-Medrano, R., Xoconostle-Cazares B., Lucas, W. J., ″Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants.″ *Development*, Vol. 126, pp. 4405–4419, 1999.

Ruiz-Medrano, R., Xoconostle-Cazares, B., Lucas, W. J., ″The phloem as a conduit for inter-organ communication.″ *Curr Opin Plant Biol*, Vol. 4, pp. 202–209, 2001.

Ruiz-Medrano, R., Kragler, F., Wolf, S., ″Signaling and Phloem-Mobile Transcripts″, in Kragler, F. and Hülskamp M. (Eds.), *Short and Long Distance Signaling*, pp. 99-131, Springer, New York, 2012.

Sasaki, T., M. Chino, et al., ″Detection of several mRNA species in rice phloem sap.″ *Plant Cell Physiol*, Vol. 39, pp. 895–897, 1998.

Schneider, T. D., and R. M. Stephens, ″Sequence Logos: A new way to display consensus sequences.″ *Nucleic Acids Res*, Vol. 18, pp. 6097–6100, 1990.

Schreiber, A. W., B. J. Shi, et al., ″Discovery of barley miRNAs through deep sequencing of short reads.″ *BMC Genomics*, Vol. 12, pp. 129, 2011.

Schulte, D., T. J. Close, et al., ″The International Barley Sequencing Consortium (IBSC) at the threshold of efficient access to the barley genome.″ *Plant Physiol*, Vol. 149, pp. 142-147, 2009.

Shin, D., S. Moon, et al., ″Expression of StMYB1R-1, a novel potato single MYB-like domain transcription factor, increases drought tolerance.″ *Plant Physiol*, Vol. 155, pp. 421-432, 2011.

Shukla, L. I., Chinnusamy, V., R. Sunkar, ″The role of microRNAs and other endogenous small RNAs in plant stress responses.″ *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, Vol. 1779, pp. 743-748, 2008.

Sjolund, R., D., ″The phloem sieve element: a river runs through it.″ *The Plant Cell*, Vol. 9(7), pp. 1137, 1997.

Steindler, C., A. Matteucci, et al., ″Shade avoidance responses are mediated by the ATHB-2 HD-zip protein, a negative regulator of gene expression.″ *Development*, Vol. 126(19), pp. 4235-4245, 1999.

Stracke, R., M. Werber, et al., ″The R2R3-MYB gene family in *Arabidopsis thaliana*.″ *Curr Opin Plant Biol*, Vol. 4(5), pp. 447-456, 2001.

Stracke, R., H. Ishihara, et al., ″Differential regulation of closely related R2R3–MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling.″ *Plant J*, Vol. 50, pp. 660–677, 2007.

Su, C. F., Y. C. Wang, et al., ″A novel MYBS3-dependent pathway confers cold tolerance in rice.″ *Plant Physiol*, Vol. 153, pp. 145–158, 2010.

Suarez-López, P., ″Long-range signalling in plant reproductive development.″ Vol. pp.43, 2005.

Sunkar, R., ″MicroRNAs with macro-effects on plant stress responses.″ In *Seminars in cell Dev Biol*, Vol. 21, pp. 805-811, 2010.

Sunkar, R., Li, Y. F., G. Jagadeeswaran, ″Functions of microRNAs in plant stress responses.″ *Trends in Plant Sci*, Vol. 17(4), pp. 196-203, 2012.

Sutton, T., U. Baumann, et al., ″Boron-toxicity tolerance in barley arising from efflux transporter amplification.″ *Science*, Vol. 318, pp. 1446–1449, 2007.

Tamura, K., D. Peterson, et al., ″MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.″ *Mol Biol Evol*, Vol. 28, pp. 2731–2739, 2011.

The International Barley Genome Sequencing Consortium (IBSC), ″A physical, genetical and functional sequence assembly of the barley genome.″ *Nature*, Vol. 491, pp. 711–717, 2012.

Tombuloglu, H., N. Semizoglu, et al., ″Boron induced expression of some stress-related genes in tomato.″ *Chemosphere*, Vol. 86, pp. 433–438, 2012.

Tombuloglu, H., Kekec, G., Sakcali, M. S et al., ″Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis.″ *Mol Genetics Genomics*, Vol. 286, pp. 1-15, 2013.

Trindade, I., C. Capitão, et al., ″miR398 and miR408 are up-regulated in response to water deficit in Medicago truncatula.″ *Planta*, Vol. 231, pp. 705-716, 2010.

Vannini, C., F. Locatelli, et al., ″Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants.″ *Plant J*, Vol. 37, pp. 115–127, 2004.

Varkonyi-Gasic, E., R. Wu, et al., ″Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs.″ *Plant Methods*, Vol. 3(1), pp. 12, 2007.

Varkonyi-Gasic, E., N. Gould, et al., ″Characterisation of microRNAs from apple (Malus domestica 'Royal Gala') vascular tissue and phloem sap.″ *BMC Plant Biol*, Vol. 10, pp. 159, 2010.

Vaucheret, H., ″Post-transcriptional small RNA pathways in plants: mechanisms and regulations.″ *Genes & Development*, Vol. 20(7), pp. 759-771, 2006.

Voinnet, O., and D. C. Baulcombe, ″Systemic signalling in gene silencing.″ *Nature*, Vol. 389, pp. 553, 1997.

Weinl, C., S. Marquardt, et al., ″Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis.″ *Plant Cell*, Vol. 17, pp. 1704–1722, 2005.

Wilkins, O., H. Nahal, et al., ″Expansion and diversification of the Populus R2R3-MYB family of transcription factors.″ *Plant Physiol*, Vol. 149, pp. 981–993, 2009.

Williams, C. E., and E. Grotewold, ″Differences between plant and animal Myb domains are fundamental for DNA binding activity, and chimeric Myb domains have novel DNA binding specificities.″ *J Biol Chem*, Vol. 272, pp. 563–571, 1997.

Xoconostle-Cazares, B., Y. Xiang, et al., ″Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem.″ *Science*, Vol. 283, pp. 94–98, 1999.

Xu, H., W. Zhang, et al., ″Gibberellic acid insensitive mRNA transport in both directions between stock and scion in Malus.″ *Tree Genetics & Genomes*, Vol. 6(6), pp. 1013-1019, 2010.

Yang, A., X. Y. Dai, et al., ″A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice.″ *J Exp Bot*, Vol. 63, pp. 2541–2556, 2012.

Yanhui, C., Y. Xiaoyuan, et al., ″The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family.″ *Plant Mol Biol*, Vol. 60, pp. 107–124, 2006.

Yanik, H., M. Turktas, et al., ″Genome-wide identification of alternate bearing-associated microRNAs (miRNAs) in olive (Olea europaea L.).″ *BMC Plant Biol*, Vol. 13(1), pp. 10, 2013.

Yau, S. K., and J. Ryan, ″Boron toxicity tolerance in crops: a viable alternative to soil amelioration.″ *Crop Sci*, Vol. 48, pp. 854–865, 2008.

Yoo, B., F. Kragler, et al., ″A systemic small RNA signaling system in plants.″ *Plant Cell*, Vol. 16, pp. 1979–2000, 2004.

Zhang, S., Sun, L., F. Kragler, ″The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation.″ *Plant Physiol* Vol. 150, pp. 378–387, 2009.

Zhang, H., J. P. Jin, et al., ″PlantTFDB 2.0: update and improvement of the comprehensive plant transcription factor database.″ *Nucleic Acids Res*, Vol. 39, pp. D1114–D1117, 2011.

Zhang, W. et al., ″Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks.″ *Plant Mol Biol*, Vol. 75, pp. 93–105, 2011.

Zhang, L., G. Zhao, et al., ″Molecular characterization of 60 isolated wheat MYB genes and analysis of their expression during abiotic stress.″ *J Exp Bot*, Vol. 63, pp. 203–214, 2012.

Zhao, B., R. Liang, et al., ″Identification of drought-induced microRNAs in rice.″ *Biochem Biophys Res Commun*, Vol. 354, pp. 585–590, 2007.

Zhao, B., L. Ge, et al., ″Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor.″ *BMC Mol Biol*, Vol. 10, pp. 29, 2009.

Zhou, L., Y. Liu, et al., ″Genome-wide identification and analysis of drought-responsive microRNAs in Oryza sativa.″ *J Exp Bot*, Vol. 61, pp. 4157–4168, 2010.

Ziegler, H., Kluge, M., ″Die Nucleinsäuren und ihre Bausteineim Siebröhrensaft von Robinia pseudoacacia L.″ *Planta*, Vol. 58(2), pp. 144-153, 1962.

APPENDIX A

HvMYB protein sequences found in this study.

>HvMYB1
PWTPEEDKILVAHIHSHGHGNWRALPKQAGLLRCGKSCRLRWINYLRPDIKRG
NFSDEEEQSIIQLHQLLGNRWSAIAARLPGRTDNEIKNVWHTHLKKRLDPSAQE
QQEEAGAAKKRKKPAAAAAAPRKRDGKVKMRNLNALTARAAAAAPVSSPER
SVSSTVTESTSTASAAAEQHGNSGSSSASASASVKEECFTSSEESEEFQIDESFWS
ETLSMPLDDLNDVCMEPHDAFGKPDGDMDYWLRLFMEGGGGSGSDDNNNHD
GALDLPQI

>HvMYB2
PWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLKRSGKSCRLRWVNYLRPDLKR
GKMTPQEESTIVQLHSLWGNRWSTIARSLPGRTDNEIKNVWHTHLKKRLDPSA
QEQQEEAGAAKKRKKPAAAAAAPRKRDGKVKMRNLNALTARAAAAAPVSSP
ERSVSSTVTESTSTASAAAEQHGNSGSSSASASASVKEECFTSSEESEEFQIDESF
WSETLSMPLDDLNDVCMEPHDAFGKPDGDMDYWLRLFMEGGGGSGSDDNNN
HDGALDLPQI

>HvMYB3
MGRAPCCEKMGLKRGPWTPEEDKILVAHIHSHGHGNWRALPKQAGLLRCGKS
CRLRWINYLRPDIKRGNFTSEEEDAIIQLHAMLGNRWSTIAARLPGRTDNEIKNV
WHTHLKKRLESSSKPSIQAEPKRNAKKPAVAASTLEGPTSEPASSLEQSLSTSSA
TDSSSSESEEFQIDDSFWSETLAMSVDSSGSGLETSDTFGADSASPSSSNDEMDF
WVTLFMQASDMHSLSQI

>HvMYB4
MGRAPCCEKMGLKRGPWTPEEDKILVAHIHSHGHGNWRALPKQAGLLRCGKS
CRLRWINYLRPDIKRGNFTSEEEDAIIQLHAMLGNRWSTIAARLPGRTDNEIKNV
WHTHLKKRLESSSKPSIQAEPKRNAKKPAVAASTLEGPTSEPASSLEQSLSTSSA
TDYSVASSLENTGSSSCSEEYQIDDSFWSETLAMSVDSFSSGMEIGDTFGADSAS
PSSSNDEMDFWVTLFMQAGDIQSLSHI

>HvMYB5
LRKGLWSPEEDEKLYNHIIRYGVGCWSSVPKLAGLQRCGKSCRLRWINYLRPDI
KRGNFTSEEEDAIIQLHAMLGNRWSTIAARLPGRTDNEIKNVWHTHLKKRLESS
SKPSIQAEPKRNAKKPAVAASTLEGPTSEPASSLEQSLSTSSATDSSSSESEEFQID
DSFWSETLAMSVDSSGSGLETSDTFGADSASPSSSNDEMDFWVTLFMQASDMH
SLSQI

>HvMYB6

LRKGLWSPEEDEKLYNHIIRYGVGCWSSVPKLAGLQRCGKSCRLRWINYLRPDI
KRGNFTSEEEDAIIQLHAMLGNRWSTIAARLPGRTDNEIKNVWHTHLKKRLESS
SKPSIQAEPKRNAKKPAVAASTLEGPTSEPASSLEQSLSTSSATDYSVASSLENT
GSSSCSEEYQIDDSFWSETLAMSVDSFSSGMEIGDTFGADSASPSSSNDEMDFW
VTLFMQAGDIQSLSHI

>HvMYB7
RKGPWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLKRSGKSCRLRWVNYLRP
DLKRGKMTPQEESTIVQLHSLWGNRWSTIARSLPGRTDNEIKNVWHTHLKKRL
QSSSKPSGQAAPKRKAKKPAVATSALEGPTSQPASSPGQSLPMSPEQSLSTSSAT
DSSSSESEEFQIDDSFWSETLAMSVDSSGSGLETSDTFGADSASPSSSNDEMDFW
VTLFMQASDMHSLSQI

>HvMYB8
RKGPWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLKRSGKSCRLRWVNYLRP
DLKRGKMTPQEESTIVQLHSLWGNRWSTIARSLPGRTDNEIKNVWHTHLKKRL
QSSSKPSGQAAPKRKAKKPAVATSALEGPTSQPASSPGQSLPMSPEQSLSTSSAT
DYSVASSLENTGSSSCSEEYQIDDSFWSETLAMSVDSFSSGMEIGDTFGADSASP
SSSNDEMDFWVTLFMQAGDIQSLSHI

>HvMYB9
MVRAPCCEKMGLKRGPWTAEEDMTLVAHIEQHGHSNWRALPKQAGLLRCGK
SCRLRWINYLRPDLKRGNFSRDEDELIVKLHSLLGNKWSLIAARLPGRTDNEIK
NYWRTHMRKKAQEEKMMAKNKASSSSPSSSDSTAATSLTTTTTCSASTPTATS
STTDAPQTSMCDEAEEEASTSATEEK

>HvMYB10
EGQLAACWGKQDDEWRKGPWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLK
RSGKSCRLRWVNYLRPDLKRGKMTPQEESTIVQLHSLWGNRWSTIARSLPGRT
DNEIKNYWRTHMRKKAQEEKMMAKNKASSSSPSSSDSTAATSLTTTTTCSAST
PTATSSTTDAPQTSMCDEAEEEASTSATEEKKAEVVQYCSAVDMDQLWNDIAA
SERYPDMMMSWGAAGHAAVAAPALEPSSPVWDLCEQDYYSLWRIDDQEYYN
KID

>HvMYB11
MDVVLQSRSSNNMAAEQEEEAELRRGPWTVDEDLTLINYISDHGEGRWNALA
RAAGLRRTGKSCRLRWLNYLRPDVKRGNFTADEQLLILDLHSRWGNRWSKIA
QHLPGRTDNEIKN

>HvMYB12
EEAAVGEEETLRRGPWTVEEDILLVNYIAAHGEGRWNSLARSAGLKRTGKSCR
LRWLNYLRPDLRRGSITPQEQLLILELHSRWGNRWSKIAQHLPGRTDNEI

>HvMYB13
PCCEKAHTNKGAWTREEDERLVAHVRAHGEGCWRSLPSAAGLLRCGKSCRLR
WINYLRPDLKRGCFSQQEEDHIVALHQILGNRWSQIASHLPGRTDNEVKNYWN
SY

>HvMYB14

CEKAHTNKGAWTKEEDQRLVAYIKAHGEGCWRSLPKAAGLLRCGKSCRLRWI
NYLRPDLKRGAFSQEEEDLIIHLHSILGNKWSQIAAQLPGRTDNEVKNFWNSFIK
KKLRQRGIDPATHKPLAPAAAAAPVSRSAVFSEAELILSSPVGEVGQHMPPLVS
AESYVYSRGSMDGAAGVHAAVGGCSDDGSLSSLSGYNNNNQTADFAGYLDA
DALHGAVIPSVSSSSTLNSMAGVSPGGAANANANATDELCCNNNSNNHHPSNS
GSGFESSTTQSTSNHHLPWLELGSISSCTEDASGAGAGADHYGAALDELKWSD
YVFDGGYQQYHQQAQCIYGDSKAAADAAAAAQFDAHGLVGINWC

>HvMYB15
PCCEKAHTNKGAWTREEDERLVAHVRAHGEGCWRSLPSAAGLLRCGKSCRLR
WINYLRPDIKRGNFTSEEEDAIIQLHAMLGNRWSTIAARLPGRTDNEI

>HvMYB16
PCCEKAHTNKGAWTREEDERLVAHVRAHGEGCWRSLPSAAGLLRCGKSCRLR
WINYLRPDLKRGNFSRDEDELIVKLHSLLGNKWSLIAARLPGRTDNEIKNYWNT
HIRRKLLGRGIDPVTHRPVTDAAGTTVSFVHPEPPKAEPVTEESKPPRCPDLNLD
LCISLPFQEVEERPPARACAKPVKMEQLQGGGGGLCFRCSILRVRGAATECSCS
SNFLGLRAGMLDFRSLEMK

>HvMYB17
KGPWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLKRSGKSCRLRWVNYLRPD
LKRGKMTPQEESTIVQLHSLWGNRWSTIARSLPGRTDNEIKNYWNTHIRRKLLG
RGIDPVTHRPVTDAAGTTVSFVHPEPPKAEPVTEESKPPRCPDLNLDLCISLPFQE
VEERPPARACAKPVKMEQLQGGGGGLCFRCSILRVRGAATECSCSSNFLGLRA
GMLDFRSLEMK

>HvMYB18
MGRAPCCEKMGLKRGPWTPEEDKILVAHIHSHGHGNWRALPKQAGLLRCGKS
CRLRWINYLRPDIKRGNFSDEEEQSIIQLHQLLGNRWSAIAARLPGRTDNEIKNY
WNTHIRRKLLGRGIDPVTHRPVTDAAGTTVSFVHPEPPKAEPVTEESKPPRCPDL
NLDLCISLPFQEVEERPPARACAKPVKMEQLQGGGGGLCFRCSILRVRGAATEC
SCSSNFLGLRAGMLDFRSLEMK

>HvMYB19
AHTNKGAWTREEDERLVAHVRAHGEGCWRSLPSAAGLLRCGKSCRLRWINYL
RPDLKRGNFSRDEDELIVKLHSLLGNKWSLIAARLPGRTDNEIKNYWRTHTRK

>HvMYB20
LPSHGEGSWRSLPKNAGLLRCGKSCRLRWINYLRDGVRRGNISKEEDDLIVKLH
ATLGNRWSLIASHLPGRTDNEIKNYWNSHLSRQIHTFRRIYTTVRDTTITVDIHK
LSAAGKRRGGRTPGQSPKSSTKKKQPVPEPVTNAKGTSSTASSPPQSDEARSAV
VDPDQNQPNNSISVSNTSDGPCSEDGTWPMVMDPVVDQTDALEANCTVDQHQ
MGLWEVSNPMDQIGILEDESEVQALLSSSVTAESGIDPGGLSQVDDLLDMDWE
GFASNLWDQPAQNGLLQPAEPQVATGSESDELESFVSWLLSDAC

>HvMYB21
MGRSPCCDKVGLKKGPWTTEEDEKLLAHIEEHGHGSWRALPSKAGLQRCGKS
CRLRWTNYLRPDIKRGNFSDEEEQSIIQLHQLLGNRWSAIAARLPGRTDNE

>HvMYB22

MGRSPCCDKVGLKKGPWTTEEDEKLLAHIEEHGHGSWRALPSKAGLQRCGKS
CRLRWTNYLRPDIKRGKFSLQEEQTIIQLHALLGNRWSAIATHLSRRTDNEIKN

>HvMYB23
MGRAPCCEKEGLKRGAWSPEEDQQLADYIAQHGHPNWRALPKHAGLLRCGKS
CRLRWVNYLSPNVKRGNFTANEEDLIIRLHQSLGNRWSVIAAQLPGRTDNEIKN
VWHTHLKKMLEDGLKPAADHDAGGCGRQKSRKQAKARSATVDAGEPYKSPP
GQSSSGLTCSTVTESAATVSSSPSDNATITSASHGHQLIKEDRFSSEAVSDDSFRS
STDVPGMIDLGAMDNDLSLPMTSPSTRSDDLDFWVKMLQEAGDIADL

>HvMYB24
MGRPPSSDENGLKKGPWTSEEDEKLMSYIQKHGHGSWRALPQLAGLNRCGKS
CRLRWTNYLKPDIKRGKFSQEEEQTILQLHTVLGNKWSAIAKHLPGRTDNEIKN
FW

>HvMYB25
MGRHSCCYKQKLRKGLWSPEEDEKLMNHITKHGHGCWSSVPKLAGLQRCGKS
CRLRWINYLRPDLKRGAFAQDEEDLIVELHAVLGNRWSQIAAQMPGRTDNEIK
NLWNSSLKKKLRQKGIDPNTHKPLTEADRSGAAPTISNERTSGSSDVNPSSTGA
LGNFGHLLSETAQSSMLLPVYDKKRPETPSLARPKVPAKELFLEQLTAGHESPSS
CRSSGPTLYFPFQQQLGYSNECGSGDGANMNSLWFNQNDFNCSTISTVMPTVSP
SALSTPMGLNLPPDNHRHGGTGIGSAPFYWDGVNPSSSGSTGSSGSNSMGFEPQ
STNSILENSVFPWTDIGVGQEKDTRVQLVEELKWPDLLHGTFAEATTAMQNQS
QSLYDDVIKAESQFNMEGICASWFQNQQPQQQLQAASDMYDKDLQRMPLSFE
HI

>HvMYB26
KKRLKRGLWSPEEDEKLMNHIAKYGNGCWSSVPKIAGLERCGKSCRLRWINYL
RPDLKRGAFAQDEEDLIVELHAVLGNRWSQIAAQMPGRTDNEIKNLWNSSLKK
KLRQKGIDPNTHKPLTEADRSGAAPTISNERTSGSSDVNPSSTGALGNFGHLLSE
TAQSSMLLPVYDKKRPETPSLARPKVPAKELFLEQLTAGHESPSSCRSSGPTLYF
PFQQQLGYSNECGSGDGANMNSLWFNQNDFNCSTISTVMPTVSPSALSTPMGL
NLPPDNHRHGGTGIGSAPFYWDGVNPSSSGSTGSSGSNSMGFEPQSTNSILENSV
FPWTDIGVGQEKDTRVQLVEELKWPDLLHGTFAEATTAMQNQSQSLYDDVIK
AESQFNMEGICASWFQNQQPQQQLQAASDMYDKDLQRMPLSFEHI

>HvMYB27
MGRAPCCDRTGLKKGPWTQEEDEKLVAYIKKHGQGNWRTLPKNAGLERCGK
SCRLRWTNYLRPDLKRGLLSDEEEKTVIDLHEQLGNRWSKIAAQLPGRTDNEIK
NHWNTHIKKKLRKMGIDPATHKP

>HvMYB28
MGRQPCCDKVGLKKGPWTAEEDQKLVSFLLSNGQCCWRAVPKLAGLLRCGK
SCRLRWTNYLRPDLKRGLLSDEEEKTVIDLHEQLGNRWSKIAAQLPGRTDNEIK
NHWNTHIKKKLRKMGIDPATHKP

>HvMYB29
CDKATVKRGPWSPEEDAMLKAYIEERGTGNNWIALPHKIGLKRCGKSCRLRW
LNYLRPNIKHGDFTPEEDSTICKLYISIGSRWSIIAAQLPGRTDNDVKNY

>HvMYB30
MGRAPCCDKASVKKGPWSPEEDATLKSYIEQNGTGGNWIALPQKIGLRRCGKS
CRLRWLNYLRPNIRHGGFSDEEDRIILSLYISIGSRWSIIAAQLPGRTDNDIKNYW
NTRLKKKLFGKQSRKDQRQHQFGRQGANGGHKEEASEGSAGNNGVAAGAYN
WHQHTMAMPPHPMPGSMVEGNFIGEGVDESIRKLLFKLGGSSFAALPAPPCLPP
PMYGEAPNFVAPSVHTAPLNEGGIHCSSVLPALELDESFQFNQVKLDGLECFFG
MGDQSMRWNEVSPLVCPNTTVASSSQGMQQYCLLEEPVDLGLQ

>HvMYB31
MGRAPCCDRAAVNRGPWSPEEDDALRDYMQRHGNTGSWITLPAKAGLKRCG
KSCRLRWLNYLRPDIRHGGFTDEEDAIIYSLYSQLGSKWSLIASQLERRTDNDV
KNHWNTKLKKRLVAAAAAFPAAPSSRRPPSFTPAAAHAHAHPSPLLPLPAPTV
KAETYTYDDFLAPTAGLHDPFAADGSSSASAAVQDPFATEGSSSASAAVQDAF
VGEGSTSASAASSGSNWSAVDNAGGFFVDFCAAGADLAVADQFLGGFYYPLD
PTLS

>HvMYB32
LVLVSYVQEHGPGNWRAVPTRTGLMRCSKSCRLRWTNYLRPGIKRGNFTDQE
EKLIVHLQALLGNRWAAIASYLPERTDNDIKNYWNTHLKRKLQAGGDAAAAK
PA

>HvMYB33
MGRPPCCDNGGASVKKGPWTPEEDIILVSYIQQHGPGNWRSVPENTGLMRCSK
SCRLRWTNYLRPGIKRGNFTQHEEGIIIHLQALLGNKWAAIASYLPQRTDNDIK
NYWNTHLKKKVKRLQQQAHPTDHFFQHT

>HvMYB34
RPSYRKGLWSPEEDQKLRDFIVRYGHSCWSTVPVKAGLQRNGKSCRLRWINYL
RPGLKHGMFSREEEETVMSLHAALGNKWSQIAQHLPGRTDNEIKNFWNSCIKK
KLRQQGIDPATHKPMASADTATAAAALPDAEEEDRKPLCPAIDGSLVPKQPAV
F

>HvMYB35
MGRHAGTGGGVQQKLRKGLWSPEEDEKLYNHIIRYGVGCWSSVPKLAGLQRC
GKSCRLRWINYLRPDLKRGSFSQQEEDAIVGLHEILGNRWSQIASHLPGRTDNEI
KNFWNSCIKKKLRQQGIDPATHKPMASADTATAAAALPDAEEEDRKPLCPAID
GSLVPKQPAVFDPFPLCVDYGAGFAEELGAANAAALYGQLCVGKEVADDDAG
FGAADYSCVLDVSENLGYGESSSNSSNWNSAPEANNALEGEALHWASESKVEP
FVGYAGGDDDKFALPCHGQQEHSMAHFDFNLE

>HvMYB36
MGCKACEKPRPSYRKGLWSPEEDQKLRDFIVRYGHSCWSTVPVKAGLQRNGK
SCRLRWINYLRPGLKHGMFSREEEETVMSLHAALGNKWSQIAQHLPGRTDNEI
KNFWNSCLKKKLRQRGIDPSTHKPISGVAGVDLDQPSKDDKPQAADGALKQQ
QLQVFDPFPAADTFGAGFDGAGMPLYDHLGGKDAVAFVDYSSVLDVSENLGY
GESSSNSSNWNSAPEANNALEGEALHWASESKVEPFVGYAGGDDDKFALPCH
GQQEHSMAHFDFNL

>HvMYB37

MGCKACEKPRPSYRKGLWSPEEDQKLRDFIVRYGHSCWSTVPVKAGLQRNGK
SCRLRWINYLRPGLKHGMFSREEEETVMSLHAALGNKWSQIAQHLPGRTDNEV
KNYWNSYLKKRVEGARSPAKSAGSGAPRSPTPSESGCGRERSVVNQQPCNSGS
SGPPESSSTADESSSLTVPGAAASIRPHAPVLPKVMF

>HvMYB38
WRKGPWTTQEDKLLLDHVAQHGEGRWNSVSKLTGLKRSGKSCRLRWVNYLR
PDLKRGKMTPQEESTIVQLHSLWGNRWSTIARSLPGRTDNEVKNYWNSYLKKR
VMQLGSNSSSNPASKNSASPELLTSMSANTELIMTSGGGGGSTATSTHDHDANI
GSSGAVSAAEPFVDHQHHQHQDAKNYVFADWMPTAAAVTAAPGPESYSMSA
HWPASTASSGNVTPSHGAFVGDQMSATSYGALQHTHQHHQQQEHQHHHQSA
VTAVGAHGGAAGMVAGGGYFDLLNMGDIYGGFTTANDDLLF

>HvMYB39
MGCRSCDKPKMNYRKGLWSPEEDQRLRDYILKHGLGCWSAVPAKAGLQRNG
KSCRLRWINYLRPGLKRGMFSQEEEDVVINLQAKLGNKWSQIAMHLPGRTDNE
VKNYWNSYLKKRVMQLGSNSSSNPASKNSASPELLTSMSANTELIMTSGGGGG
STATSTHDHDANIGSSGAVSAAE

>HvMYB40
MEMKERQRWRPEEDAILRSYVRQYGPREWNLVAQRMNAPLDRDAKSCLERW
KNYLRPGIKKGSLTDDEQRLVIRLQAKHGNKWKKIAAEVPGRTAKRLGKWWE
VFKEKQQREIRDSRRPPPEPSPDERGRYEWLLENFAEKLV

>HvMYB41
GGAKPSSLCPRGHWRPGEDEKLRQLVEKYGPQNWNSIAEKLEGRSGKSCRLR
WFNQLDPRINKRPFTEEEEERLLAAHRVHGNKWALIARHFPGRTDNAVKNHW
NCSLKRRLADVGVAEEEERPCKRASVTPESTSGSGSDRSDLSNGGVLDFGLGQ
VYRPLARTGRFEPADCAMSQRHEVELEEEAEDPLTSLSLSLPGTDAHGFHHDSS
HSHLHQPSPSPSPPPAPAPTSYPFSPEFAAVMQEMVLDEVRRYISGVGCSANLPS
MPQVVDGVMRAAAERAG

>HvMYB42
SSSTTNTSEGGAKPSSLCPRGHWRPGEDEKLRQLVEKYGPQNWNSIAEKLEGRS
GKSCRLRWFNQLDPRINKRPFTEEEEERLLAAHRVHGNKWALIARHFPGRTDN
AVKNHWHVVRARRSRERCRLLAKAASSTFPSYSYSGGAQLDFTGASAGSFCFG
FSKPGGGGFFGSPPAAAAAPSSTPVLFNGYGVGASGSKSLLSRYSSYVDGGKQP
AAPSSLSIAFSSPPSIEALALDGRGGHDHHRRKDYHVDGDEPLKTKDAPPFIDFL
GVGV

>HvMYB43
MGAESECDRIKGPWSPEEDEALRRLVERHGARNWTAIGRGIPGRSGKSCRLRW
CNQLSPQVERRPFTAEEDAAILRAHARLGNRWAAIARLLRGRTDNAVKNHWN
SSLKRKLATATGGTAWEEASDSGDSPRPCKRASPGPESPTGSDRSELSHGSGQV
FRPVPRPGGFDAISADVRPPPPRQDEDPITSLSLSLPGLDHGFHHDSARSHFQELS
PSPRSPSPPPAPQPSSSSYPFSGDLVAAMQEMIRAEVCKYMSGVGLPAGCGAGA
VGEACIPQLVEGVMRAAAERVGVMTRQ

>HvMYB44

VMAACRGGGGGGGKDVDRIKGPWSPEEDDALQRLVGRHGARNWSLISKSIPG
RSGKSCRLRWCNQLSPQVEHRPFTPDEDDTILRAHARFGNKWATIARLLSGRTD
NAIKNHWNSTLKRKYSSSSSASASASASATASLTHP

>HvMYB45
PWSPEEDAMLTNLVMRDGARNWTSIARGIPGRSGKSCRLRWCNQLDPQVKRK
PFTEEEDQMIISAHAIHGNKWASIAKLLVGRTDNAIKNHWNSTLRRRYCTGTRC
TRSASAEQPIREMSRAASEERWPLSGPSS

>HvMYB46
WSPEEDGALRQLVELHGARNWTAIGRGVPGRSGKSCRLRWCNQLSPGVERRPF
TADEDAVIARAHARLGNRWAAIARLLRGRTDNAVKNHWHVIMARRCRERMR
LKSNRGGMPAAAASTTAAAAEKDESPRNTTGGEKPRLPEANTMAALLHKYRR
EFAAPFAISHHSSKEGYCSSTNEDTNRSVEFYDFLQVNVSSSDTKCGSSIEEQEE
NRDDDDKQAEGQVALIDFMEVGAS

>HvMYB47
RRKRAPWSPVEDAQLLNILNEIGEKWTEIGRMIGGRTGKQVRDRYLYTLKPDIN
KNKWTKKEDSLLHSLYIELGNKWGEIAQHLPGRTGNQVKNRFISKFRRTLS

>HvMYB48
MATGPDLTSSSSADAAAAAASSAAKKDRHIVSWSAEEDDVLRAQIAHHGTDN
WAVIATQFKDKTARQCRRRWYNYLNTECKKGGWSREEDMLLCDAQKLLGNK
WTEIAKVVPGRTDNAVKNRFSTLCKRRAKDDELFEENSTVCSNANAKRVLTQT
GGLTCGAPSSSPPIKNMSSCNPDFKENLAPNMKSFGQEKSIRHDSRQPLSRICPD
NQSVNIVKTQSLVTKTSTKQLHGEEQSCVKHEGNFLKRNDPKLATLLQQADLL
SSLATKVNTENTSQSMDEAWQKLQHHLVKKDDNDMSESSMSGTASLLDDLDD
LIVDPYENEEEDEQKSREQNGVTSQIAPDQIMDNCPVDQITEENSLCGNTLSSSM
EPCPVAEILAYVNLGEAAEDMGLHCMEYSSPALAAQAQQAKADAEILASVDLS
ESAEAQAQQAKADAEIPASVDLSEAAEGSWSQFMEYMSPVHTVLHAEGDAET
PASLKLSEAAEGSLPQCMEHMSPAHTL

>HvMYB49
NVSGTPHDTAKHCHGEELGNIGGQLCKNDGHSFKESCSITNSEVLTRVKWSEEE
DKMLTQMINKHGLKNWQTVAHAIPGRSAPQCRQRWRYKIDSAINKEAWSEQE
ELRLIRAHQIYGTKWREMVKHFPGRTNGAIKEYWRGPMKRKLNSYLSSGLLEQ
FPDILENLSGTQNNSLDILKGTKVS

>HvMYB50
SGTPHDTAKHCHGEELGNIGGQLCKNDGHSFKESCSITNSEVLTRVKWSEEEDK
MLTQMINKHGLKNWQTVAHAIPGRSAPQCRQRWRYKIDSAINKEAWSEQEEL
RLIRAHQIYGTKWREMVKHFPGRTNGAIKEYWRGPMKRKLNSYLSSGLLEQFP
DILENLSGTQNNSLDILKGTKVS

>HvMYB51
MRIMIKGGVWKNTEDEILKAAVMKYGKNQWARISSLLVRKSAKQCKARWYE
WLDPSIKKTEWTREEDEKLLHLAKLMPTQWRTIAPIVGRTPSQCLERYEKLLDA
ACAKDENYEPNDDPRKLRPGEIDPNPESKPARPDPVDMDEDEKEMLSEARARL
ANTRGKKAKRKAREKQLEEARRLASLQKRRELKAAGIDNRHKKRKRKGIDYN
AEIPFEKRPPPGFYDTVGEDRPLEHVQFPTTIEELEGRRRVDVEAQLRKQDIAKN

KILQRQDAPAAIMQANKLNDPEAVTRRSKLMLPPPQISDHELEEIAKMGNAGDP
ALAEELGEGSTATRTLLANYSQTPRLGMTPLRTPQRTPGGKGDAIMMEAENLA
RLRESQTPLLGGDNPELHPSDFSGVTPRKKEIQTPNPMATPLASPGPGVTPRIGL
TPSRDGNAFGLTPKGTPFRDELHINEEVEMQDSAQLELRRQAELRRGLRSGFASI
PQPKNEYQIVMPPITEEKEEAEERIEEDMSDRLARERAEEQARQEALLRKRSKV
LQRSLPRPPAASVEILRQSLIKGGETRSTFVPPTSLEQADELINEELLRLLEHDNA
KYPLDEQTQREKKKGSKRQANGAAFVPEIEGFDEHELTEASSMVEEEIQYLRVA
MGHENESFEDFVKSHDACQEDLMFFPTNNSYGLASVAGNADKISALQHEFEMV
KKRMDDEAKKASRLEQKIKLLTQGYQARAAKLGSQIQDTFKQMNTAATELEC
FQELQKQEQMAAAYRVKNLSDEVNKQKALERTLQSRYGDLLSGYQSIHEQLEE
HKRLLRLQAETIEAENRA

APPENDIX B

DECLARATION STATEMENT FOR THE ORIGINALITY OF THE THESIS, FURTHER STUDIES AND PUBLICATIONS FROM THESIS WORK

A.1 DECLARATION STATEMENT FOR THE ORIGINALITY OF THE THESIS

I hereby declare that this thesis comprises my original work. No material in this thesis has been previously published and written by another person, except where due reference is made in the text of the thesis. I further declare that this thesis contains no material which has been submitted for a degree or diploma or other qualifications at any other university.

Signature:
Date: January, 25, 2014

A.2 FURTHER STUDIES

Further studies related to this thesis work can be done in the following areas:

(a) Functional studies about the roles of miRNA upon stress conditions.
(b) Cloning of genes to the model organisms.
(c) Characterization of MYB transcription factors for the functions on plants.

A.3 PUBLICATIONS FROM THESIS WORK

Academic Journals

- Tombuloglu, H., Kekec, G., Sakcali, M. S., & Unver, T. (2013). Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. *Molecular Genetics and Genomics*, 1-15.

Conference Proceedings

- Tombuloglu, H., Kekec, G., Sakcali, M. S., & Unver, T. Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. *2. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi,* 15-18 November, 2012, Antalya, Turkey.

Posters

- Tombuloglu, H., Kekec, G., Sakcali, M. S., & Unver, T. Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. *New Model Systems for Linking Evolution and Ecology*, 1-4 May, 2013, Heidelberg, Germany.

CURRICULUM VITAE

CONTACT INFORMATION
Hüseyin TOMBULOĞLU
Fatih Üniversitesi, Biyoloji Bölümü, 34500 Büyükçekmece, Istanbul, Turkey
Phone: (0212) 866 33 00-2064
Email: htombuloglu@fatih.edu.tr; hsyntm@gmail.com

EDUCATION

- **PhD**, Fatih University, BiotechnologyIstanbul-Turkey, 2014
- **PhD**, Gebze Institute of Technology, Chemistry Biochemistry (Not graduated)-2011, Kocaeli Turkey
- **MS**, Fatih University, Dept of Biology-2010, Istanbul-Turkey
- **BS**, Istanbul University, Molecular Biology and Genetics.-2007, Istanbul
- University of Groningen, Department of Eukaryotic Microbiology-2006, Groningen- the Netherlands

PROFESSIONAL EXPERIENCE

- Researcher TUBITAK-MAM (Marmara Research Center), Oct. 2008- Oct. 2009
- Teaching Assistant Fatih University, Dec. 2007
- Exchange Student University of Groningen, Mar. 2006- Aug. 2006

RESEARCH PROJECTS

- Zeytinde (Olea Europaea L.) Yağ Biyosentezi Mekanizmasında Rol Alan miRNA'ların Tanımlanması Ve Karakterizasyonu, TUBITAK, Oct 2013-Oct 2014, Ana Yönetici Amount: 27500
- Bitkilerde uzak organ iletişiminde bora bağlı anlatım farklılığı gösteren mobil miRNA' ların belirlenmesi , Üniversite Araştırma Proje Fonu, Aug 2013-Aug 2014, Ana Yönetici Amount: 10000
- Arpada (Hordeum vulgare L.) bora karşı direnç mekanizmasının transkriptom düzeyinde incelenmesi , TUBITAK, Apr 2011-Apr 2012, Araştırmacı Amount: 24100
- Bor Kirliliğinin Mısır (Zea mays L.) ve Fasülye (Phaseolus vulgaris L.) Üzerindeki Etkilerinin Fizyolojik ve Biyokimyasal Olarak İncelenmesi ,Üniversite Araştırma Proje Fonu, Nov 2010-Nov 2011, Araştırmacı Amount: 8000
- Bor toksisitesine direnç gösteren genlerin mRNA differential display tekniği kullanılarak belirlenmesi,, Üniversite Araştırma Proje Fonu, Jun 2009-Jun 2011, Araştırmacı Amount: 8000 TL
- Investigation of active boron uptake mechanism of plants and generation of boron tolerant alfaalfa cultivars that will be able to grow up contaminated soils.

- BOREN (National Boron Research Institute), Apr 2009-Apr 2011, Araştırmacı Amount: 49 822 TL
- Cloning of AtBor4 gene to Medicago sativa to generate boron tolerant plant cultivar, Üniversite Araştırma Proje Fonu, Jan 2008-Jan 2010, Araştırmacı Amount: 15 000 TL
- Investigation of heavy metal and hydrocarbone polluted soil remediation by using plants, algeas and microorganisms., TUBITAK - KAMAG, Jan 2006-Jan 2010, Araştırmacı Amount: 1 500 000 TL

AWARDS AND HONORS

- Yurt Dışı Bilimsel Etkinliklere Katılma Desteği, TÜBİTAK, 01/07/2009
- Yurt Dışı Bilimsel Etkinliklere Katılma Desteği, TUBITAK, 01/02/2012
- Young Researcher Fellowship, European Molecular Biology Laboratories-EMBL, 04/05/2013

PUBLICATIONS

Books/Book Chapters

- "Boron and Plants", (Munir Ozturk, Serdal Sakcali, Salih Gucel, and Huseyin Tombuloglu) in Plant adaptation and Phytoremediation, Springer, 01/08/2010, pp. 275-312.

Academic Journals

- Ertuğrul Filiz, Hüseyin Tombuloğlu, İbrahim Koç, "Genome-wide identification and analysis of growth regulating factor (GRF) genes in Brachypodium distachyon, in silico approaches", Turkish Journal of Biology, Vol. In press, Jan. 2014.

- Kekec, G., Sakcali, MS, Uzonur, I, Alpsoy, L., Tombuloglu, H., "RAPD Analysis for Detecting Genotoxic Effects of Boron on Maize (Zea Mays L.)", Toxicology and Industrial Health, Vol.(In press). Jan. 2014,

- Ertugrul Filiz, İbrahim İlker Özyiğit, Hüseyin Tombuloğlu, İbrahim Koç, "In silico comparative analysis of LEA (Late Embryogenesis Abundant) proteins in Brachypodium distachyon L.", Plant Omics Journal, Vol. 6, No. 6, Nov. 2013, pp. 433-440.

- Ertugrul Filiz, Huseyin Tombuloglu, Ibrahim Ilker Ozyigit, "Genome wide analysis of IQ67 domain (IQD) gene families in Brachypodium distachyon", POJ-Plant Omics Journal, Vol. 6, No. 6, Nov. 2013, pp. 425-432.

- Tombuloglu Huseyin, Kekec Guzin, Sakcali Serdal, Unver Turgay.,"Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis", Molecular Genetics and Genomics, Vol. 288, No. 3-4, Apr. 2013, pp. 141-155.

- Huseyin Tombuloglu; Nihan Semizoglu; Serdal Sakcali; Guzin Kekec ,"Boron induced expression of some stress-related genes in tomato", Chemosphere, Vol. 86, Jan. 2012, pp. 433-438

Conference Proceedings

- Tombuloglu, H., Kekec, G., Sakcali, M. S., & Unver, T. Transcriptome-wide identification of R2R3-MYB transcription factors in barley with their boron responsive expression analysis. *2. Ulusal Moleküler Biyoloji ve Biyoteknoloji Kongresi,* 15-18 November, 2012, Antalya, Turkey.
- Hüseyin Tombuloğlu, M.Serdal Sakçalı, Fahri Akbaş, "GENERATING Medicago sativa L. CULTIVARS THAT ARE TOLERANT TO HIGH LEVELS OF BORON", 4th International Boron Symposium, Eskişehir/Türkiye, Oct. 2009,
- Hüseyin Tombuloğlu, Güzin Kekeç, M.Serdal Sakçalı, Fahri Akbaş,"CLONING OF AtBor4 GENE TO Medicago sativa L. (Leguminosae-Fabacea) TO GENERATE BORON TOLERANT PLANT CULTIVAR", 14th Europan Congress of Biotechnology , Barcelona-Spain, Sep. 2009, New Biotechnology, 25S, pp. 299.
- Hüseyin Tombuloğlu, M.Serdal Sakçalı, Fahri Akbaş, "CLONING OF AtBor4 GENE TO Medicago sativa L. (Leguminosae-Fabacea) TO GENERATE BORON TOLERANT PLANT CULTIVAR", International Conferance on Plants & Environmental Pollution, Kayseri/ Türkiye, Jul. 2009, International Conferance on Plants & Environmental Pollution, pp. 85.

Affiliations

- EMBL-European Molecular Biology Laboratories
- Ulusal Proteomik Derneği (Proteomics Society of Turkey)
- European Federation of Biotechnology