DIVERSITY OF microRNAs AND GENES TOWARDS DEVELOPMENT OF DROUGHT-TOLERANT WHEAT

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Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Sabancı University

January 2015

DIVERSITY OF microRNAs AND GENES TOWARDS DEVELOPMENT OF DROUGHT-TOLERANT WHEAT

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DATE OF APPROVAL: 30/12/2014

ABSTRACT

DIVERSITY OF microRNAs AND GENES TOWARDS DEVELOPMENT OF DROUGHT-TOLERANT WHEAT

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PhD Thesis, 2015

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Keywords : microRNA, *Triticeae*, drought, wheat improvement

World is threatened by global warming resulting in elevated incidence of drought, the primary cause of yield loss in wheat. Domestication of wheat species, followed by years of breeding for maximum yield, has eradicated genetic diversity in the long run and leading to the loss of valuable alleles for drought stress tolerance in today's elite cultivars. Cellular responses to stress conditions usually involve intermingled, complex networks of gene interactions. Therefore, understanding the molecular basis of stress responses in wheat and related species is highly challenging but also, crucial. In the first project, we introgressed drought-related genomic regions to elite germplasm, providing potentially high drought tolerant bread wheat. Although the capacity of plants to tolerate drought is largely coded in their genomes, it is of equal importance to understand the efficient activation of drought response mechanisms by elaborating regulation of a complex network of gene interactions. Integral to these stress responses are, undoubtedly, microRNAs, which act as post-transcriptional regulators of gene expression. In the second project, we identified and investigated microRNAs and their target genes in wheat and related species and further characterized their responses to drought. Comparative analyses of microRNA repertoires and microRNA target functions across several wheat species indicate conserved or unique patterns of drought tolerance mechanisms. microRNA repertoires reported here will be convenient for further studies expanding our understanding of gene regulation across wheat and related species and the role of microRNAs in drought tolerance.

ÖZET

KURAKLIĞA DİRENÇLİ BUĞDAY GELİŞTİRİLMESİNE YÖNELİK GENLERİN VE mikroRNA'LARIN ÇEŞİTLİLİĞİ

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Biyolojik Bilimler ve Biyomühendislik Bölümü
Doktora Tezi, 2014

Prof. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar kelimeler: mikroRNA, *Triticeae*, kuraklık, buğday geliştirilmesi

Dünya genelinde buğday verim kaybının en önemli nedeni olan kuraklık, son yıllarda küresel ısınma ile büyük oranda artmıştır. Buğday türlerinin ehlileştirilmesini takiben yüksek verime yönelik ıslah çalışmaları, uzun vadede gen çeşitliliğini önemli ölçüde daraltmış, günümüzde kullanılmakta olan elit buğday çeşitlerinde, kuraklık direnci açısından önemli alellerin kaybına neden olmuştur. Bitkilerde, stres koşullarına hücresel yanıtlar çoğunlukla karmaşık gen etkileşim ağları ile sağlanır. Bu nedenle buğday ve ilgili türlerde stres moleküler mekanizmalarının aydınlatılması oldukça zor, ancak bir o kadar da önemlidir. Bu projede ilk olarak, kuraklığa ilişkin önemli genomik bölgeleri elit türlere aktararak, potansiyel olarak kuraklığa dirençli ekmeklik buğday geliştirdik. Kuraklığa direnç kapasitesi, büyük ölçüde bitkinin genomu tarafından belirlense de, karmaşık gen etkileşim ağlarının regülasyonu incelemek, kuraklık mekanizmalarının etkili bir şekilde aktivasyonunu anlamak açısından genom bazında yapılan çalışmalar kadar önemlidir. Bu bağlamda, gen ekspresyonunun post-transkripsiyonel olarak regülasyonunda görev alan mikroRNA adlı moleküllerin incelenmesi büyük önem taşımaktadır. Projenin ikinci kısmında, buğday ve ilgili türlerde bulunan mikroRNAları ve bu moleküllerin hedef genlerini saptadık ve kuraklığa ilişkin olarak karakterize ettik. mikroRNA repertuarlarının ve fonksiyonlarının karşılaştırmalı analizi, korunmuş veya türe özgü kuraklık direnç mekanizmalarına işaret etmektedir. Bu çalışmada saptanan mikroRNAlar, buğday ve ilgili türlerde gen regülasyonunun ve mikroRNAların kuraklık direncindeki rolünün aydınlatılması kapsamında ileri çalışmalar açısından önem taşımaktadır.

With all my heart,

To all PHD students

ACKNOWLEDGEMENTS

First of all, I would like to thank to my supervisor Prof. Dr. Hikmet Budak. His advices throughout my doctroral years were priceless for me. He has influenced me in ways that transcend academics and his voice will guide me throughout my career and my life.

I would also like to thank each member of my thesis committee: Prof. Dr. Yusuf Menceloğlu, Assoc. Prof. Levent Öztürk, Assoc. Prof. Ali Koşar and Assist. Prof. Bahar Soğutmaz Özdemir. I am grateful for their valuable comments.

I wish to express my gratitude to my lab members: Dr. Stuart James Lucas for his assistance and Dr. Meral Yüce, Bala Anı Akpınar, Reyyan Bulut, Zaeema Khan, Naimat Ullah, Deniz Adalı, İpek Özdemir, Burcu Alptekin and Babar Hussain for their support. Additionally I am very grateful to Barış Tümer for his time and valuable technical assistance. I would also like to thank Yusuf Tutuş and Mustafa Atilla Yazıcı for their assistance during my experiments in the greenhouse and growth chambers. I especially want to thank Yusuf Tutuş for all his time and guidance.

I would also like to thank the Scientific and Technological Research Council of Turkey for the financial support they have provided during my doctoral years.

Finally and most important of all I would like to express my sincerest gratitude to my dear mother and father for their infinite support, patience and faith in me. I especially want to thank to my ever young sister, Selda Kantar and my dear friend with the kindest heart, Reyyan Bulut. I owe all my achievements to their unconditional support and love.

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ABBREVIATIONS

^{o}C	Degree Celcius
5DS	5D chromosome short arm
5DL	5D chromosome long arm
µ	Micro
µPC	MicroPC
ABA	Abscisic acid
AGO	Argonaute
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide-Nucleotide BLAST Algorithm
BLASTP	Protein-Protein BLAST Algorithm
BLASTX	Nucleotide-Protein BLAST Algorithm
bp	Basepair
cal	Calory
cDNA	Complementary DNA
CP1	C-Terminal Domain Phosphatase-Like1 Protein
CV	Coefficient of Variation
DCL1	Dicer-Like 1 Protein
DH	Doubled Haploid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
DREB	Dehydration-Responsive Element-Binding Protein
dsRNA	Double Stranded RNA
emPCR	Emulsion Polymerase Chain Reaction

En/Spm	Enhancer/Suppressor-Mutator Transposable Element Family
EST	Expressed Sequence Tag
F	Filial
FAO	Food and Agricultural Organization of the United Nations
FL	Fraction Length
g	Gram
gDNA	Genomic DNA
G+C	Guanine-Cytosine
HEN1	HUA ENHANCER1
HSP	Heat Shock Protein
HST	HASTY
HYL1	HYPONASTIC LEAVES 1
k	Kilo
l	Liter
LEA	Late Embryogenesis Abundant Protein
LINE	Long Interspersed Nuclear Element
LTR	Long Terminal Repeat
m	Mili
M	Molar
m7Gppp	5' 7-methylguanosine cap
MAS	Marker-Assisted Selection
MFE(ΔG)	Minimum Folding Free Energy
MFEI	Minimal Folding Free Energy Index
$MgCl_2$	Magnesium Chloride
min	Minute
MIR	microRNA Encoding Gene

miRNA	microRNA
miRNA*	microRNA Passenger Strand
mol	Mole
mRNA	Messenger RNA
MuDR	Mutator Transposable Element Family
n	Nano
NCBI	National Center for Biotechnology Information
ncRNA	Non coding RNA
NGS	Next Generation Sequencing
NIL	Near Isogenic Line
nt	Nucleotide
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PIWI	P-element induced wimpy testis
PlantGDB	Plant Gene DataBase
poly(A)	Polyadenylate
ppm	Parts Per Million
pre-miRNA	Preliminary microRNA
pri-miRNA	Primary microRNA
qRT-PCR	Quantitative-Real Time Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RIL	Recombinant Inbred Line
RISC	RNA-induced silencing complex
RLM-RACE	RNA Ligation Mediated Rapid Amplification of complementary DNA ends
RNA	Ribonucleic Acid
RNase	Ribonuclease

ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Reverse Transcription PCR
s	Second
SE	SERRATE
siRNA	Small Interfering RNA
sRNA	Small RNA
SSR	Simple Sequence Repeat
Taq	Thermus Aquaticus
TcMar	TcMariner Transposable Element family
TGH	TOUGH
tRNA	Transfer RNA
U	Unit
UTR	Untranslated Region

1.

1.INTRODUCTION

With increasing demands on land and water, simply relying on existing resources is not possible and we need to produce more from the available resources. This is not the first time world has faced such a challenge. Approximately half a century ago, population growth threatened to overtake food production and at that point, it was discovered that semi-dwarf mutants of wheat produced much more grain than their taller relatives. A series of research, development and technology transfer initiatives so-called Green Revolution has led to steady annual increases in grain production, in which selective breeding for yield and other important traits played an important role (Kantar, Lucas, and Budak 2011).

However, this growth may no longer be adequate to meet future demand (Tester and Langridge 2010). World is threatened by global warming resulting in increased incidence of environmental stresses, making stabilizing yields as much of a challenge as increasing them. Climate change has detrimental consequences particularly for crops which hold great economic value (Habash, Kehel, and Nachit 2009). Drought, arguably the most significant single abiotic stress factor is currently increasing worlwide, effecting progressively more arable land and impacting agricultural production.

Wheat and its related species are of great importance, constituting the primary sources of food and feed consumption. However, domestication of wheat species,

followed by years of cultivation, genetics and breeding practices has considerably narrowed gene pools of today's elite cultivars. These practices introduce an artificial selection pressure for yield, ultimately eradicating genetic diversity, resulting in the loss of valuable alleles for drought stress tolerance. Ironically, the semi-dwarfism trait that drastically improved grain yields 50 years ago makes wheat more vulnerable to drought in many cases. Therefore, it is crucial to take initiatives for the next Green Revolution to develop wheat yielding high even under water-limited environments.

For improvement of drought tolerant wheat varieties, understanding the molecular basis of stress responses in modern crops and their drought tolerant wild progenitors; and effective transfer of this molecular information to breeding are crucial. However, this is also highly challenging since cellular responses to stress usually involve intermingled networks of molecular interactions. Although the capacity of plants to tolerate drought is coded from their genomes, it is of equal importance to understand the efficient activation of drought response mechanisms by elaborating the complex system of gene regulation. Integral to this stress regulation are, undoubtedly, microRNAs (miRNAs) which act as post-transcriptional regulators of gene expression.

Here, we identified and investigated miRNAs and their target genes in different *Triticaea* species and further characterised their responses to drought. Comparative miRNA repertoires reported here hold valuable information regarding to conserved or unique patterns of molecular response to stress. This information will be convenient for further studies expanding our understanding of gene regulation across *Triticaea* and the role of miRNAs in drought tolerance. Besides, also in this project, in an effort to improve drought tolerance in modern wheat with high yield characteristics, we introgressed a recently identified drought related genomic region from South Austrialian cultivars to European elite germplasm. This new wheat genotype potentially high yielding under water-limited environments is available for further physiological and environment targeted field testing.

2.

2.OVERVIEW

2.1. *Triticeae*

The tribe *Triticeae* of the subfamily *Pooideae* in the monocototyledonous grass family (*Poaceae*), includes nearly 400 perennial and 100 annual taxa. *Triticeae* has played an extremely valuable role in human civilization and it includes species that are indispensable for human welfare. It encompasses forage and lawn grasses as well as several agriculturally important domesticated major crops from the genera *Hordeum* (barley), *Triticum* (wheat) and *Secale* (rye), which are traditionally cultivated in the temperate zone. These species have been used as staple food and beverages in various ways throughout the history of mankind. *Triticeae* species have a complex evolutionary history being subjected to domestication. A phylogenetic tree of *Triticeae* species with divergence time estimates is shown in Figure 1 (Middleton et al. 2014). *Triticeae* tribe has a basic chromosome number of seven and comprises diploids ($2n=2x=14$), as well as species with varying degrees of polyploidy up to duodecaploids ($2n=12x=84$). Allopolyploidization, a cytogenetic process during hybridization resulting in the presence of complete chromosome sets of both parents in the progeny, has been and still is the major driving force on this tribe's evolution. Hence, this natural process has been utilized to artificially create species through intergeneric or interspecific hybridization, increasing the genetic variability within the tribe. For instance, Triticale (*Triticosecale)*, a currently commercial crop was synthesized by artificial hybridization to develop a crop with high grain quality and quantity of wheat, and superior stress tolerance of rye. Elucidation of molecular mechanisms underlying differential yield and stress characteristics of *Triticeae* genera, species, subspecies and cultivars and their

integration into breeding programmes is crucial for further improvement of their agronomic performance and ameliorate the effects of climate change (Middleton et al. 2014; Wang and Lu 2014; Wang et al. 2010).

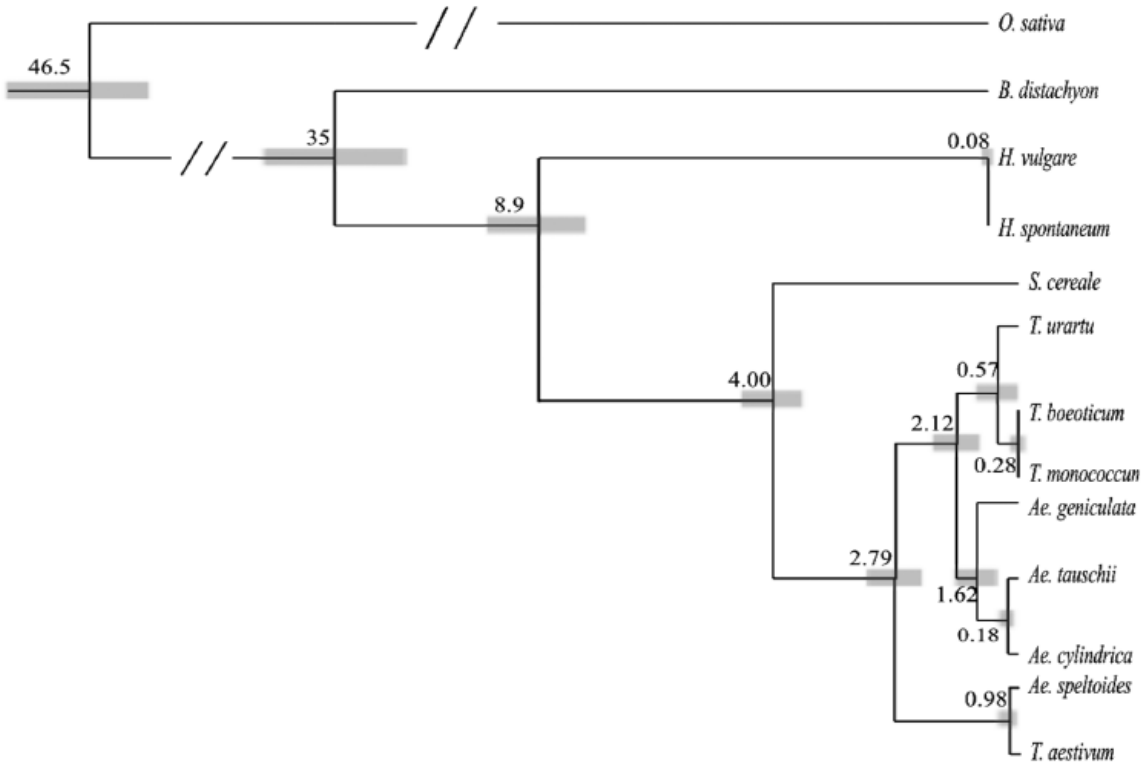

Figure 1 Phylogenetic tree of *Triticeae* species with divergence time estimates.
Divergence times are shown as numbers at anchors in million years.
Gray boxes represent standard deviations of divergence times.
Analysis was performed using chloroplast sequences and tree was drawn according to the divergence of *Oryza sativa* and *Brachypodium distachyon* as anchor points.
(O.sativa: Oryza sativa, B. distachyon: Brachypodium distachyon, A.geniculata: Aegilops geniculata, A. cylindrica: Aegilops cylindrica, A. speltoides: Aegilops speltoides, A. tauschii: Aegilops tauschii, T. aestivum: Triticum aestivum, T. monococcum: Triticum monococcum, T. boeticum: Triticum boeticum, T. urartu: Triticum urartu, H. spontaneum: Hordeum spontaneum, H. vulgare: Hordeum vulgare, S. cereale: Secela cereale)
(Middleton et al. 2014)

2.1.1. Barley as a Major Crop

Cultivated barley (*Hordeum vulgare* L; 2*n*=2*x*=14), domesticated from its wild progenitor *Hordeum vulgare ssp. spontaneum* 10,000 years ago in the Near East Fertile Crescent, is among the founder crops of agriculture (Badr et al. 2000). It is currently the fourth most abundant cereal grain globally both in terms of agricultural area (approximately 5 million hectar) and annual production (approximtely 145 million metric tons) based on Food and Agriculture Organization of United Nations (FAO) statistics of 2013 (http://faostat.fao.org). Geographic distribution of average barley production between years 1993-2013 is shown in Figure 2 a. This crop is majorly used as an animal feed (75%) and also malted for beverage production (20%). It is also recently becoming popular as human food owing to the high content of soluble diatary fibre in its grain, which reduces the risk of multiple serious human diseases. In addition to its agricultural importance, due to its diploid, relatively low complexity genome and self-pollinating nature, it has been traditionally considered as a *Triticeae* model species for genetics and breeding. Hence, currently, an extensive amount of barley genetic resources and tools are available including molecular markers, genetic maps, large collection of expressed sequences, bacterial artificial chromosome (BAC) clone constructs, mutant collections, large scale of double haploids and barley transformation techniques. Recently, a physical map representing 95% of the barley genome (haploid barley genome: 5.1 gigabasepairs) was also developed. Barley genome was estimated to contain approximately 30,400 genes. Through homology to annotated genomes of *Pooaceae* model species 26,159 genes were determined with high confidence, of which 24,154 were positioned on the physical/genetic scaffold (Mayer et al. 2011, 2012). Full annotation of barley genome is expected to be available in the near future (http://webblast.ipk-gatersleben.de/barley/index.php). As its sequence databases are enriched, large collections of germplasm containing barley elite varieties and wild accessions are also available. These are undoubtedly rich resources for crop improvement since barley is more stress tolerant than its close relative wheat, being widely adapted to diverse environmental conditions (Kantar, Unver, and Budak 2010; Mayer et al. 2012; Mrízová et al. 2014)

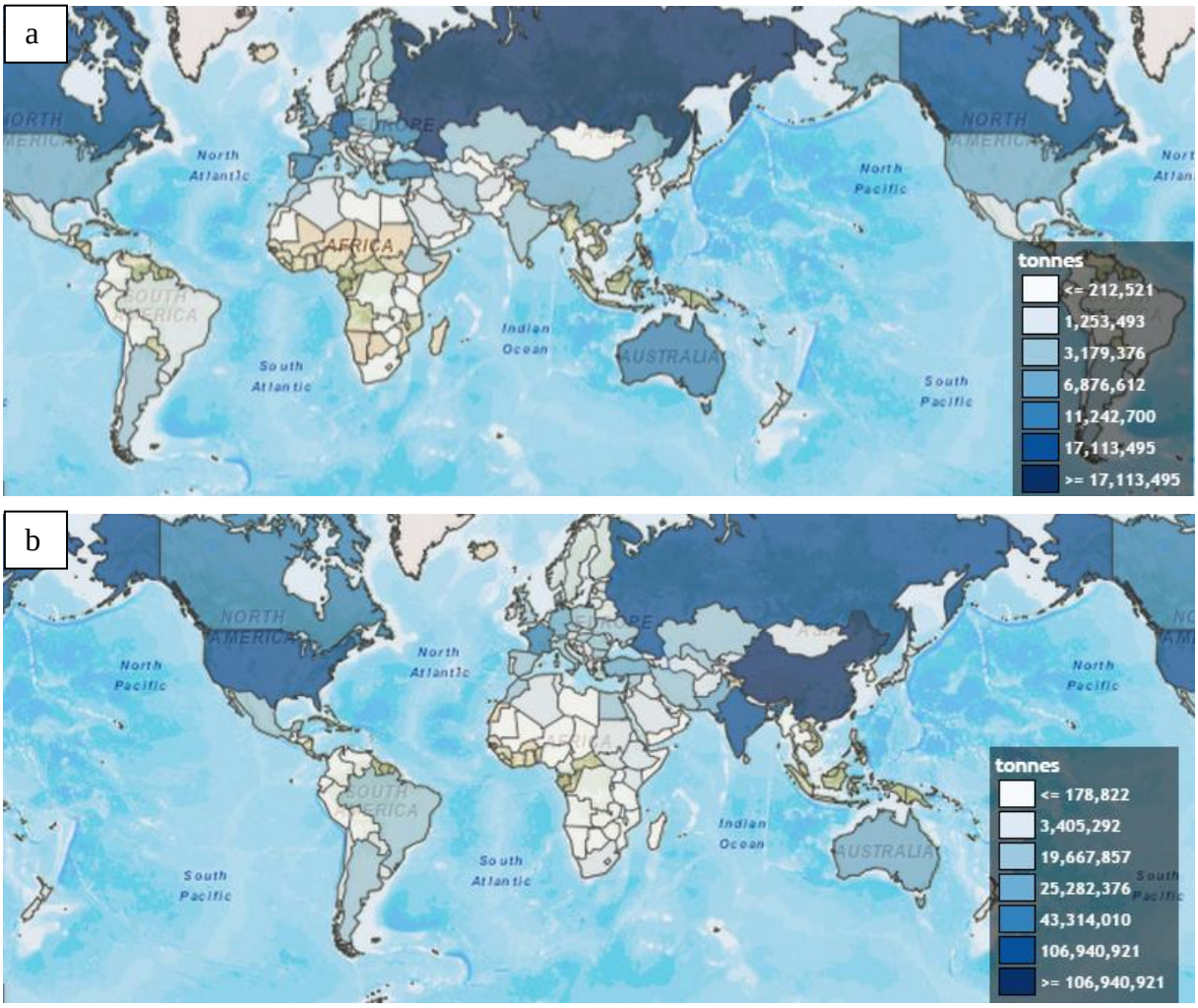

Figure 2 Geographic distribution of average production of (a) barley (b) wheat between years 1993-2013. (http://faostat.fao.org)

2.1.2. Wheat as a Major Crop

Wheat is currently the most extensively grown crop in the world covering 30% of the agricultural area (approximately 218 million hector) used for cereal cultivation. With a global annual production of over 713 million tones, wheat is the third most abundantly produced crop, following maize and rice (based on FAO statistics of 2013 ; http://faostat.fao.org). Geographic distribution of average wheat production between years 1993-2013 is shown in Figure 2 b. Wheat is a fundamental source for protein, vitamins and minerals for human food consumption, providing almost 20% of the human dietary energy supply in calories (http://www.fao.org, 2011). Wheat cultivation and domestication has been directly associated with the spread of agriculture. Cultivated

wheat refers mainly to two types: hexaploid bread wheat (*Triticum aestivum* L.; AABBDD, 2*n*=6*x*=42) accounting for about 95% of world wheat production, and the tetraploid durum wheat (*T. turgidum* ssp. *durum;* AABB, 2*n*=4*x*=28) accounting for the remaining 5%. Domesticated tetraploid durum is one of the oldest cultivated cereal species in the world and its domestication from wild emmer wheat (*T. turgidum* ssp. *dicoccoides*; AABB, 2*n*=4*x*=28) in the Near East Fertile Crescent, dates to approximately 10,000 year ago. Allohexaploid bread wheat is originated from a hybridization between cultivated allotetraploid emmer wheat and diploid goat grass (DD, *Aegilops tauschii*) approximately 8,000 years ago in the Near East Fertile Crescent. The three diploid genome progenitors: *Triticum urartu* (AA), *Aegilops tauschii* (DD) an unknown BB progenitor (possibly *Sitopsis* section species similar to *Aegilops speltoides*) radiated from a common *Triticeae* ancestor 2.5-4.5 million years ago and AABB tetraploids arose less than 0.5 million years ago (Brenchley et al. 2012; Feldman 2001; Kurtoglu, Kantar, and Budak 2014).

Large (~17 gigabasepairs), highly repetitive (80%) wheat genome with three homeologous, but divergent subgenomes has for long been considered refractory to sequencing. Despite its complexity, recently a great progress has been achieved in elucidating the genomic background of wheat with the development of next generation sequencing technologies. Hence, wheat genome was estimated to harbour 94,000-96,000 genes, of which two-thirds were assigned to subgenomes through comparisons to diploid ancestral genomes (Brenchley et al. 2012). Besides, the recent advent of chromosome flow sorting technique rendered the study of individual chromosomes, resolving the problem of identifying which sub-genome a particular feature belongs to. Draft survey sequencing of bread wheat chromosomes is currently available (Berkman et al. 2011; Hernandez et al. 2012; Lucas and Budak 2012; Tanaka et al. 2014; Vitulo et al. 2011). Projects are now underway to develop chromosome based physical maps, so far reported for 1AL, 1AS, 1BS, 1BL, 3B and 6A (Breen et al. 2013; Lucas et al. 2013; Paux et al. 2008; Philippe et al. 2013; Poursarebani et al. 2014; Raats et al. 2013). These physical maps will serve as substrates to the end of completing the reference sequences of all bread wheat chromosomes (International Wheat Genome Sequencing Consortium, www.wheatgenome.org) as produced recently for 3B (Choulet et al. 2014). Increasing knowledge on wheat genome structure has accelerated discovery of genes underlying important agronomic traits to the end of improving this major crop.

2.2. Drought as a Major Abiotic Stress Factor

To meet the demands of the ever-growing population, world food production needs to be doubled by the year 2050 (Qin, Shinozaki, and Yamaguchi-Shinozaki 2011; Tilman et al. 2002). Abiotic stresses, as the primary causes of agricultural loss worldwide, are estimated to result in an average yield loss of more than 50% for most crops (Akpinar, Lucas, and Budak 2013; Boyer 1982; Bray, Bailey-Serres, and Weretilnyk 2000; Qin et al. 2011). Global environmental warming, with the prospect of increasing environmental stresses threatens the world's food supply, making stabilizing yields as much of a challenge as increasing them (Kantar, Stuart J Lucas, et al. 2011; Nevo and Chen 2010). Drought in crop production results from a shortage of water in the root zone (Nevo and Chen 2010; Salekdeh et al. 2009). Constant and sproadic periods of drought is currently the most prominent and widespread abiotic stress, accounting for a significant portion of the yield loss resulting from abiotic factors and effecting more than 10% of arable land (Akpinar et al. 2013; Bray et al. 2000; Kantar, Stuart J Lucas, et al. 2011). Geographic distribution of water withdrawal for agricultural use between years 1990-2010 (http://faostat.fao.org) is shown in Figure 3.

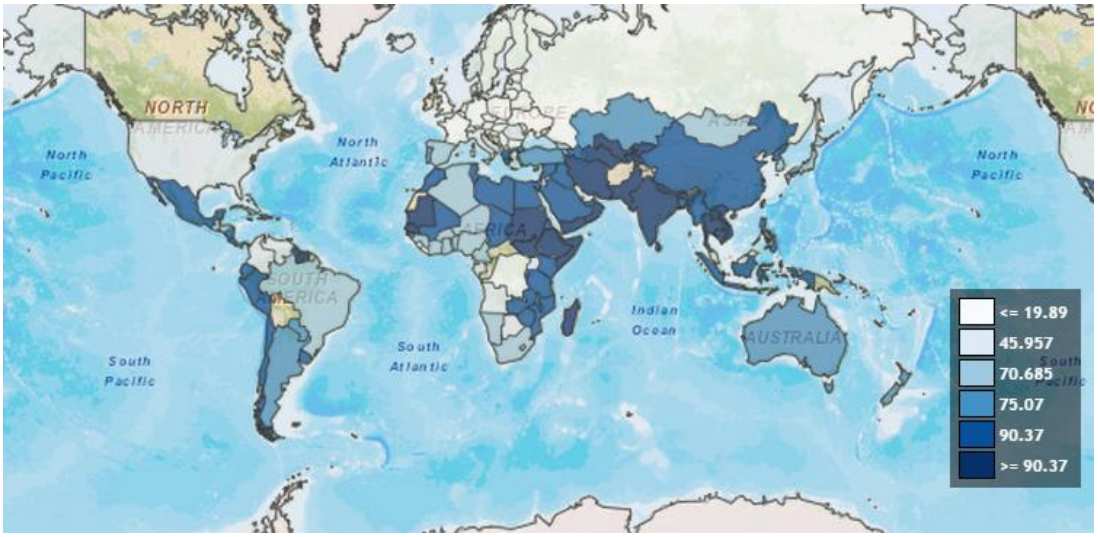

Figure 3 Geographic distribution of percentage of water withdrawal for agricultural use between years 1990-2010. (http://faostat.fao.org)

2.2.1. Drought Tolerance

Drought tolerance is the ability of a plant to access soil water and use it efficiently to live, grow and reproduce satisfactorily under conditions of limited water supply or under periodic conditions of water deficit (Fleury et al. 2010; Kantar, Stuart J Lucas, et al. 2011; Munns et al. 2010; Richards et al. 2010; Turner 1979). Tolerance strategies include resistance mechanisms, which enable plants to survive osmotic stress, and avoidance mechanisms, which prevent plants' exposure to dehydration through growth habits like deeper rooting for better access soil water, or shortened growth span through faster development and maturation (Fleury et al. 2010; Kantar, Stuart J Lucas, et al. 2011; Nevo and Chen 2010). Most plants have developed strategies to cope with drought stress having evolved in habitats with limited water availibility (Kantar, Stuart J Lucas, et al. 2011). However, modern crop species, have drastically lost their tolerance to environmental stresses, including drought through the process of domestication, followed by centuries of cultivation (refer to Section 2.2.3) (Dubcovsky and Dvorak 2007; Kantar, Stuart J Lucas, et al. 2011; Nevo and Chen 2010; Nevo 2004; Reynolds and Condon 2007; Tang, Sezen, and Paterson 2010).

2.2.2. Molecular Biology of Drought

The capacity of plants to tolerate drought depends largely on the drought adaptation mechanisms within their genomes, and how efficiently these mechanisms are activated when plants are exposed to stress. Few agronomic traits are controlled by single genes or isolated biological pathways. Likewise, genetic control of plant response to drought is a complex trait controlled by an intermingled network of gene interactions regulated at multiple levels and highly effected by environmental factors. Elucidation the complete molecular basis of drought response and tolerance is highly challenging, yet crucial.

Drought has a multitude of detrimental effects on plant cellular function. Drought responses of plants include attenuated growth and suppression of core metabolism.

Exposure to drought is followed by a decrease in osmotic potential and cellular dehydration, causing reduced cytosolic and vacuolar volumes. With the suppression of core metabolism, reactive oxygen species (ROS) (e.g. singlet oxygen and hydrogen peroxide) are highly accumulated majorly from chloroplasts and to some extend from mitochondria, causing oxidative stress, resulting in cellular and protein damage (Ergen et al. 2009; Kantar, Stuart J Lucas, et al. 2011)

Plant response to drought aims to minimize these harmful effects for continuation of plant survival, growth and reproduction. This includes stimulation of multiple signal transduction cascades consisting of a network of protein interactions mediated by reversible phophorylation (e.g. mitogen activated protein kinases, sucrose nonfermenting-like kinases, phosphotases) and release of secondary messengers (e.g. phospholipid and calcium signalling) triggering cellular and physiological changes. Following dehydration, compatible solutes, sugars, sugar alcohols, amino acids, or other nontoxic molecules (e.g. proline, glycine betaine), are highly accumulated in the cytoplasm and are believed to confer osmotic adjustment without interfering with the metabolism (Barnabás, Jäger, and Fehér 2008; Bartels and Sunkar 2005; Valliyodan and Nguyen 2006). Likewise, levels of chemical (e.g. ascorbate, carotenoids) and enzymatic (e.g. superoxidase dismutase, catalase) antioxidants, which cope with oxidative damage by scaveging ROS, are also drought induced (Shinozaki and Yamaguchi-Shinozaki 2007). To ameloriate the effects of oxidative damage, late embryogenesis abundant proteins (LEAs) (e.g. dehydrin) and molecular chaperones like heat shock proteins (HSPs) also accumulate during osmotic stress aiding in functional protection of essential proteins (Mahajan and Tuteja 2005; Wang, Vinocur, and Altman 2003). Drought response is a complex process, in which several other cellular mechanisms have been implicated including signalling through molecules like salicyclic acid, or nitric oxide; as well as regulation of transport through aquaporins and ion channels.

Activation of various cellular mechanisms for triggering drought response demands the synthesis of new proteins and degradation of existing ones that are not or less essential in this environment (Barnabás et al. 2008; Bartels and Sunkar 2005; Mahajan and Tuteja 2005). These alterations in expression profiles is regulated elaborately in multiple levels: transcriptional, post-transcriptional, post-translational. Transcriptional regulation of drought-induced gene products is achieved through

activation of several transcription factors and trancriptional regulators; and abscisic acid (ABA)-dependent and -independent pathways are two well-established transcriptional regulatory circuits induced by drought. Plant genes involved in drought response are also known to be regulated at the post-transcriptional level by the action of miRNAs (refer to Section 2.2.4). Similarly, a number of post-translational modifications (e.g. ubiquitination, small ubiquitin-like modifier-ylation, isoprenylation) with different cellular roles have also been shown to contribute to regulation in response to drought (Ergen et al. 2009; Kantar, Stuart J Lucas, et al. 2011).

2.2.3. Wild Progenitors of Domesticated Crops

As the availability of water for agriculture is becoming limited, as explained in Section 2.2, there is growing emphasis on the need to identify and dissect novel drought-response mechanisms to utilize in the genetic improvement of cultivated crops for stress tolerance. Domestication of crops, followed by centuries of cultivation has considerably narrowed the gene pools of today's elite cultivars, drastically reducing their stress tolerance. Common agricultural practices favor breeding under tightly controlled conditions, which introduces an artificial selection pressure for production yield, which eradicates the crop germplasm diversity in the long run, and leads to the loss of valuable alleles for stress tolerance. For development of high yielding cultivars under stress conditions, investigation of naturally occuring relatives of modern crops hold great potential as these drought- resistant ancestors are valuable sources harbouring advantegous stress adaptation and tolerance pathways. As progenitors of cultivated wheat and barley: *T. dicoccoides* and *H. spontaneum* have recently gained prominenence as genetic resources for novel drought mechanisms (Akpinar et al. 2013; Ergen et al. 2009; Kantar, Lucas, and Budak 2011; Nevo and Chen 2010).

2.2.3.1. Wild Emmer Wheat

T. dicoccoides is the tetraploid progenitor of both bread wheat and domesticated tetraploid durum wheat, as noted in Section 2.2.1. It is thought to have originated in north-eastern Israel and the Golan and diversified into the Near East Fertile Crescent, through adaptation to a spectrum of ecological conditions. As revealed by the analysis of allozyme and DNA marker variations, wild emmer wheat populations exhibit a high level of genetic diversity, showing significant correlation with environmental factors. Hence *T: dicoccoides* gene pool harbours a rich allelic repertoire of agronomically important traits (Dong et al. 2009; Fahima et al. 1999, 2002; Nevo and Beiles 1989; Nevo et al. 1982; Wang et al. 2008) including drought (Peleg et al. 2005, 2008). Some of its accessions are even fully fertile under extreme arid environments (Nevo et al. 1984) and compared to durum wheat, several thrive better under water limitation (Ergen and Budak 2009; Peleg et al. 2005). Two highly promising drought tolerant varieties originating from southeastern Turkey where the climate is characterized by long drought periods are TR39477 and TR38828 evident by morphological observations and physiological measurements in response to slow dehydration stress (Ergen and Budak 2009). Although *T. dicoccoides* genome sequence is currently unavailable, information regarding transcript, protein and/or metabolite profiles of Turkish (drought tolerant TR39477; drought sensitive TTD-22) and Israelean (drought tolerant: Y12-3 and drought sensitive: A24-39) varieties is swiftly accumulating, revealing pathways unique to dehydration tolerant wild emmer wheat (Budak, Akpinar, et al. 2013; Ergen and Budak 2009; Ergen et al. 2009; Krugman et al. 2010, 2011). Some of the drought related gene candidates discovered in these studies (integral transmembrane protein inducible by tumor necrosis factor-α; dehydration responsive element binding factor 1, autophagy related protein 8) were even further functionally characterized in relation to their roles in dehydration (Kuzuoglu-Ozturk et al. 2012; Lucas, Dogan, and Budak 2011; Lucas, Durmaz, et al. 2011). With its high drought tolerance and compatibility in crossing with durum and bread wheat (Feldman and Sears 1981), wild emmer wheat is an important resorvoir of novel drought-related mechanisms and highly suitable as a donor for improving drought tolerance (Budak, Kantar, and Kurtoglu 2013; Nevo and Chen 2010; Peng, Sun, and Nevo 2011, 2011; Xie and Nevo 2008).

2.2.4. microRNAs

Not all transcribed genes are translated into proteins and the majority of the eukaryotic transcriptome consists of non coding RNAs (ncRNAs), which have diverse, significant cellular functions. These ncRNAs are classified into several subcategories, one of which is small RNAs (sRNAs), molecules that exert RNA-mediated silencing of genes (Yi et al. 2014). A well-defined class of sRNAs is miRNAs: endogenous 18-25 nucleotide-long molecules generated from double stranded RNA regions of hairpin-shaped precursors and function as post-transcriptional regulators of gene expression (Zhang and Wang 2015). The first miRNA to be identified was *Caenorhabditis elegans* Lin-4 (Lee, Feinbaum, and Ambros 1993) and in the decade following its discovery, miRNAs were shown to be ubiquitous to other invertebrates and vertebrates. In 2002, these small molecules were also found to be present in a variety of plant species (Llave, Kasschau, et al. 2002; Llave, Xie, et al. 2002; Mallory et al. 2002; Marker et al. 2002; Park et al. 2002; Reinhart et al. 2002).

Since then, miRNAs has been attracting huge attention and miRNA-related research has currently become one of hottest research topics of molecular biology. miRNAs have been shown to be involved in a variety of physiological processes in relation to plant growth, development and response to stress (Zhang and Wang 2015). With the increasing evidence on their important cellular roles, major efforts have been put into developing advanced methods for their identification. Hence with the advance of new technologies and bioinformatics tools, the number of miRNA related publications have boosted in the last decade (refer to Section 2.2.4.2). The known miRNA repertoires of plants is continuously growing, enriching miRNA repositories like miRBase (http://www.mirbase.org) (Griffiths-Jones 2004; Griffiths-Jones et al. 2008; Kozomara and Griffiths-Jones 2014). The current version of miRBase (version 21, June 2014) contains 6,995 preliminary miRNA (pre-miRNAs) and 8,508 mature miRNA sequences from 74 plant species (refer to Section 2.2.4.1).

2.2.4.1. microRNA Biogenesis and Mechanism of Silencing

miRNA biogenesis is a highly complex mechanism, however it has been the most intensively investigated area in the miRNA field through the last decade and its general machinary is extensively dissected. A simplified model of miRNA biogenesis and function in plants is given in Figure 4 (Zhang, Pan, Cobb, et al. 2006). Plant miRNAs are encoded as independent transcription units by their own genes (*MIRs*) at diverse intergenic and much less frequently genic locations. miRNA biogenesis starts with the transcription of a long single stranded primary transcript with the action of RNA polymerase II, which is recruited to MIR promoters by the general transcriptional coactivator, Mediator. The generated transcript is referred as the primary miRNA (pri-miRNA), stabilized by the addition of a 5' 7-methylguanosine (m7Gppp) cap and 3' polyadenylate (poly(A)) tail to avoid potential degradation and folded into an imperfect double stranded RNA (dsRNA) hairpin through base pairings. Pri-miRNAs are than sequentially processed into short mature miRNA sequences in multiple steps. First, stem-loop hairpin structures that form within pri-miRNA are cleaved near the base of their stem generating smaller fold-back stem loop intermediates termed pre-miRNA. Pre-miRNAs are further cleaved to produce duplexes, which include both miRNA guide strand and miRNA passenger strand (miRNA*) with 2 nucleotide 3' overhangs. These processes are controlled through the action of Dicer-like nucleases (members of Ribonuclease (RNase) III endonucleases); in fact majorly DICER-LIKE 1 (DCL1). Several other proteins are also involved in the regulation of miRNA processing. These include RNA binding proteins that interact with DCL1 in the *microprocessor* complex (TOUGH (TGH), SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1)) possibly required for DCL1 recruitment and/or function. Phosphorylation is an important regulatory mechanism during processing and C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 (CPL1) is known to maintain the phosphorylated state of HYL1. Several of the above mentioned miRNA biogenesis proteins (DCL1, TGH, SE, HYL1 and CPL1) were recently found to colocalize in nucleolus-associated bodies along with miRNA precursors, highlighting these subnuclear loci as dicing centers (Budak, Khan, and Kantar 2014; Kumar 2014; Rogers and Chen 2012, 2013; Zhang and Wang 2015).

After dicing is completed in the nucleus, generated miRNA/miRNA* duplex, is transported into the cytoplasm by HASTY (HST). The 3' nucleotides of miRNA and miRNA are 2'-O-methylated in the duplex by the methyltransferase HUA ENHANCER 1 (HEN1) and subsequently the duplex is separated by helicase. miRNA* is degraded and mature miRNA enters the ribonucleoprotein complex known as the RNA-induced silencing complex (RISC), directing it to the target complementary messenger RNA (mRNA). After their loading to RISC, plant miRNAs can presumably exert gene regulation through different mechanisms. Target mRNA cleavage' is the most commonly adopted mechanism by plant miRNAs, in which the RNaseH-like P-element induced wimpy testis (PIWI) domain of ARGANOUTE (AGO) proteins form an RNaseH-like fold with a slicer endonuclease activity and cleaves RNA targets that are complementary to the loaded guide strand. Two other mechanisms of miRNA exerted gene regulation, well-established in animals are (1) translational inhibition, in which regulation is achieved by hampering ribosome movements along the mRNA and (2) mRNA decay, in which deadenylation of 3' poly(A) tail or decapping of 5' end of the mRNA results in its destabilization and progressive degradation. Although to date there is no biochemical proof for the presence of these alternative mechanisms in plants, several lines of evidence support the existence of slicing independent miRNA exerted regulation. Hence, some *Arabidopsis* mutants were found to be selectively impaired in miRNA-mediated gene repression at the protein, but not mRNA levels and this repression was further shown to be insensitive to inhibition of AGO1 slicing (Budak et al. 2014; Kumar 2014; Rogers and Chen 2012, 2013; Zhang and Wang 2015).

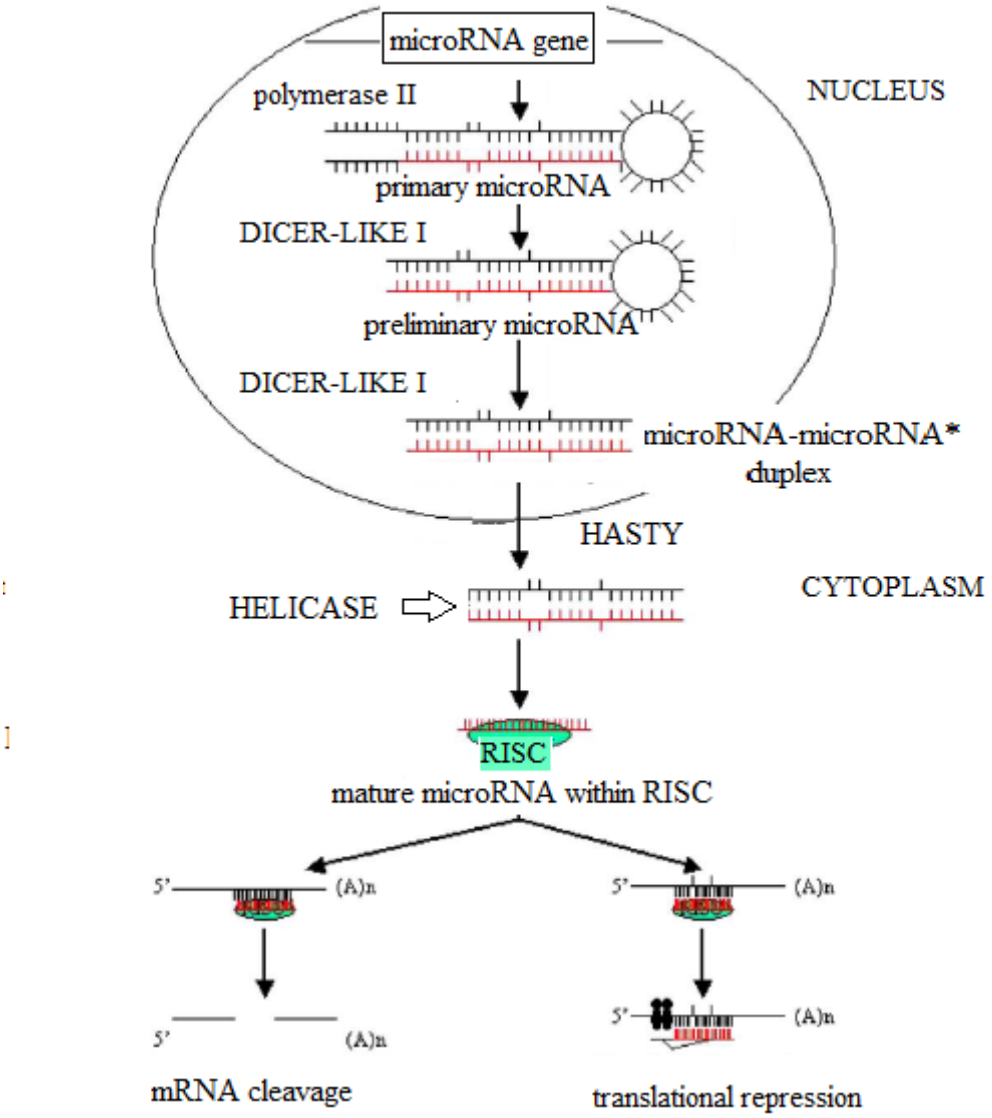

Figure 4 Simplified model of microRNA biogenesis and function in plants.
(RISC: RNA-induced silencing complex, (A)n: polyadenylate tail)
(modified from Zhang, Pan, Cobb, et al. 2006)

2.2.4.2. Methods of microRNA Identification

As evidence on the significant physiological roles of miRNAs in cellular processes increases, the techniques for their identification are becoming progressively more sophisticated. One of the conventional techniques for miRNA identification has been forward genetic screening, which has the advantage of providing information in relation to miRNA function. Yet, being cost and time ineffective, as well as incidental, this method has enabled the discovery of only a limited number of miRNAs. In order to

overcome some of the shortcomings of this approach, high-throughput transcriptomics techniques: hybridization-based platforms (microarrays) and deep-sequencing of sRNA libraries, were implemented for miRNA detection. These techniques hold an additional advantage of wide-scale comparison of miRNA profiles in distinct tissues, at different developmental stages and in plants grown under different conditions (Budak et al. 2014).

Bioinformatics tools, as essential components of high-throughput experimental sRNA data interpretation aside, are also extensively utilized as principal and less resource-intensive strategies for miRNA discovery in species where genome or transcriptome sequence information is available. While, these methods are automated and new bioinformatics tools are developed to process high-throughput data with high specificity, sequence datasets of many plants, which may serve as comprehensive inputs for miRNA prediction are swiftly accumulating. Hence, widespread application of next generation sequencing (NGS) technologies has highly contributed to miRNA identification, boosting related computational and high-throughput experimental studies (Budak et al. 2014).

Still, functional implications of especially computationally identified miRNAs necessitate verification through other experimental procedures, such as quantitative real time polymerase chain reaction (PCR) (qRT-PCR), Northern blotting, RNA Gel blots, or splinted-ligation based detection. Additionally, in order to place miRNAs on a broader context, the knowledge on their respective target(s) is a key to understand the functional relevance of miRNAs at the cellular level; thus, miRNA data should always be evaluated with the corresponding target data. miRNA targets can be identified either through computational approaches (web-based tools like psRNATarget, plantgrn.noble.org/psRNATarget) (Dai, Zhuang, and Zhao 2011), and/or experimental methods like RNA ligation-mediated rapid amplification of complementary DNA (cDNA) ends (RLM-RACE) or its high-throughput application, 'degradome sequencing' (Thomson, Bracken, and Goodall 2011).

2.2.5. Identification of Drought-related Molecules in *Triticeae*

Prior to focusing on individual drought-related components for improvement of wheat or its related species, identification of sets of probable dehydration stress-related molecules or quantitative trait loci (QTLs) is necessary. One method for identifying potential markers for stress tolerance is QTL mapping of yield related traits under drought prone environments. Another means of identifying such markers is transcript, protein, and/or metabolite profiling to monitor changes in response to dehydration, or comparing differential repertoires of plants with varrying tolerance to drought. In the long run, these markers can aid in screening cultivars for drought tolerance/sensitivity and/or improvement of drought tolerance in wheat and its related species (Budak, Kantar, et al. 2013).

2.2.5.1. Drought-related Quantitative Trait Locus Identification in *Triticeae*

Elaborating drought tolerance, effected by multiple loci necessitates the identification of related QTLs. Although QTL cloning requires a large investment in relation to resources, technology, and time, QTL discovery provides great advantages to the end of developing better yielding cultivars (refer to Section 2.2.6). Recent progresses in functional and comparative genomics have boosted resources such as BACs, sequence data, molecular markers and bioinformatic tools, rendering construction of molecular maps, which are utilized in QTL mapping. Several appropriate wheat and barley mapping populations including recombinant inbred lines (RILs) and near isogenic lines (NILs) have been established, available to be used in mapping and fine mapping of candidate regions of traits, prior to their positional cloning (Budak, Kantar, et al. 2013).

Recently, through linkage analysis and association mapping, several QTLs related to particular components of drought response were identified in mapping populations, derived from crosses of *T. aestivum*, *T. durum*, *H. vulgare* and their wild progenitors, *T. dicoccoides* and *H.spontaneum* (Budak, Kantar, et al. 2013; Fleury et al. 2010; Nevo and Chen 2010). However, despite the substantial research efforts on QTL mapping and

the recent acceleration in positional cloning with the increase in availablity of genetics resources (Collins, Tardieu, and Tuberosa 2008), to date only a limited number of studies have succeeded in positional cloning of barley and wheat QTLs and none in the context of drought. The genomic regions associated with individual QTLs are still very large and usually inappropriate for screening in breeding programmes (Fleury et al. 2010).

Yield being the highest priority trait to breeders, to date the majority of QTLs were mapped through assessment of yield and yield components under water-limited environments. However, this research is complicated due to yield and drought both being complex traits controlled by multiple genes and showing environmental interactions. QTLs identified from one environment may not be consistent with those discovered in another, hence, large scale phenotyping trials in multiple fields, taking into account the environmental differences hold great value (Fleury et al. 2010). In this regard, recent studies performed on doubled haploid (DH) populations derived from crosses between two southern Australian bread wheat cultivars: RAC875 and Kukri hold great importance. These parentals lines have differential tolerance to water deficit and have been extensively evaluated in relation to yield, yield component traits and morpho-physiological mechanisms under different severities of drought (Izanloo et al. 2008). Hence, in a number of studies, RILs established from these parentals, were investigated under a variety of environments including multiple field and year combinations in distinct seasonal conditions. Multienvironmental analysis was performed in both advantageous and adverse conditions, including nonirrigated fields. Linkage maps were constructed revealing several genomic regions or gene blocks for grain yield and quality; other yield components and morphophysiological traits (Bennett, Izanloo, Edwards, et al. 2012; Bennett, Izanloo, Reynolds, et al. 2012; Bennett, Reynolds, et al. 2012; Bonneau et al. 2013). QTLs identified in these studies are highly promising in eventually finding their way into practical wheat breeding programmes in relation to drought.

2.2.5.2. Drought-related microRNA Identification in *Triticeae*

With the application of next-generation deep sequencing and advanced bioinformatics, as outlined in Section 2.2.4.2, miRNA-related studies have expanded to non-model plants including *Triticaea* species (Budak et al. 2014; Zhang and Wang 2015). In the past years, the number of identified miRNAs in bread wheat (Budak et al. 2014) and barley (Hackenberg et al. 2013; Kruszka et al. 2014; Ozhuner et al. 2013) has dramatically increased. Since miRNAs play a critical role in almost all biological processes, most of these studies focussed on particular phases and aspects of plant development (Houston et al. 2013; Tang et al. 2012) and/or plant response to a variety of abiotic (Ozhuner et al. 2013; Xin et al. 2010) or biotic (Liu et al. 2014; Xin et al. 2010) stresses (Zhang and Wang 2015).

As partial miRNA repertoires accumulate from disparate studies, this growth should be succeeded with careful assessment of available data in the genomic, subgenomic and transcriptomic contexts. At this point, this aspect is particularly important for bread wheat, for which currently a huge amount of partial and disparate miRNA data is available (Budak et al. 2014). On the other hand, despite the huge progress in *T. aestivum*, miRNA research in *T. turgidum* species has lagged behind. The knowledge on *T. turgidum* miRNAs is currently limited to those identified in a couple of studies in *T. turgidum* ssp dicoccon (Li et al. 2014) and durum wheat (Dryanova, Zakharov, and Gulick 2008; Kenan-Eichler et al. 2011). Hence, in the current state of research, it is crucial that actions are taken for large-scale discovery of miRNAs in *T. turgidum* species.

miRNAs provide a unique strategy for crop improvement, yet their effective utilization in breeding for stress tolerance requires the determination of novel miRNA-regulated pathways valuable for conferring stress tolerance. To this end, comparative evaluation of stress regulated miRNA reportoires of elite varieties and their stress tolerant progenitors is crucial for revealing conserved and distinct stress related mechanisms. Although, miRNA repertoires of wheat and barley have been investigated extensively in relation a variety of stress factors, suprisingly related research conducted in the context of drought has been limited. Only very recently, a number of drought

responsive miRNAs in modern species: bread wheat (Gupta et al. 2014) and barley (Hackenberg et al. 2014; Kapazoglou et al. 2013) were reported. With the exception of our's group's study on wild emmer wheat (Kantar, Stuart J. Lucas, et al. 2011), no miRNA related research has has been yet conducted in wild species of the genus *Triticeae*.

2.2.6. Improvement of Drought-Tolerant Cultivars

Recent advances in molecular biological, functional, and comparative tools open up new opportunities for the molecular improvement of modern wheat. Recently developed techniques enable faster identification and characterization of drought-related components. Natural variants of modern species harbor a large repertoire of potential drought-related genes and hold a tremendous potential for wheat improvement. Introduction of drought-related components of wheat can be performed either with breeding through marker-assisted selection or transgenic methods (Budak, Kantar, et al. 2013; Nevo and Chen 2010).

Transgenic methods are adventageous since they enable the transfer of only the desired loci from a source organism to elite wheat cultivars, avoiding possible decrease in yield due to the cotransfer of unwanted adjacent gene segments. Components integral to several stress related pathways are the most appealing targets for crop improvement, since their introduction can potentially enhance tolerance to multiple environmental threats (Budak, Kantar, et al. 2013). Hence, overexpression of a number of such proteins (cotton and *Arabidopsis* Dehydration-Responsive Element-Binding proteins (DREBs) and barley LEA) were observed to increase drought tolerance in wheat under laboratory and experimental field conditions (Guo et al. 2009; Hoisington and Ortiz 2008; Pellegrineschi et al. 2004). Similarly being central to regulation of multiple stress related and developmental pathways, miRNAs are also promising candidates for genetic improvement of wheat. For instance, a combined approach of artifical miRNA and artificial target mimicry was recently developed and succeeded in improving panicle exsertion in rice, resulting in higher yields (Chen et al. 2013). Overall, transgenics hold

great potential for improvement of drought tolerant common commercial crops, the current methods used for wheat transformation are laborious and time consuming, but new transgenics methodologies are currently being developed (Chauhan and Khurana 2011).

A more established method for crop improvement is molecular breeding, which utilizes molecular markers for the screening of specific traits across cultivars. Loci that are targeted in marker-assisted selection (MAS) are most often derived from QTL mapping studies of quantitative traits. MAS is most often performed based on physiomorphological characteristics related to yield under drought conditions. Most commonly used molecular markers in such a context include SSR (simple sequence repeat) markers (Budak, Kantar, et al. 2013). For instance, SSR marker, *gwm*312 is being routinely used in durum breeding programs (James, Davenport, and Munns 2006) to transfer and select for the presence of sodium (Na^+) exclusion (Nax) genes, which are involved in sequestration of Na^+ in the vacuole compartment, enhancing osmotic adjustment capability and ameliorating the negative effects of drought (Brini et al. 2005). Currently the major challange to MAS is that most of the potential drought related genes which can be used for selection purposes (e.g. DREBs) belong to large gene families (Wei et al. 2008). Hence, identification and successful isolation of a single drought-related loci is complicated by the members of the same family with high sequence similarity and in the case of bread wheat its complex, polyploid genome. However, in the very near future, completion of wheat reference genome will pace the identification of specific loci and the development of markers to be used in selection during breeding processes (Witcombe et al. 2008). Recent increase in sequence availability has already contributed to the discovery of drought-related QTLs and provided several high quality genetic markers for breeding (Bennett, Izanloo, Edwards, et al. 2012; Bennett, Izanloo, Reynolds, et al. 2012; Bennett, Reynolds, et al. 2012; Bonneau et al. 2013).

Up until now, no drought tolerant wheat or barley genotype has been produced through conventional and molecular approaches, which has found its way to the farmer's field. However, it is not unreasonable to predict in the following decades, such cereals will be transferred to the fields as a common commercial crop owing to recent efforts and advances.

3.

3.MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Fertilizers and Enzymes

Chemicals, fertilizers and enzymes used in this research are listed in Appendix A.

3.1.2. Molecular Biology Kits

Molecular biology kits used in this research are listed in Appendix B.

3.1.3. Plant Material

Two lines of the bread wheat nulli-tetrasomic series lacking 5D chromosome (N5D-T5A and N5DT5B) and four homozygous lines from the bread wheat group-5 chromosome deletion series (5DS-2, 5DS-5, 5DL-5, 5DL-7) were obtained from Kansas State University. Australian bread wheat cultivars (Kukri and RAC875) were obtained

from Australian Centre for Plant Functional Genomics. Elite cultivars (bread wheat variants: Tosunbey and Bolal and barley variant: Bülbül-89) were obtained from Field Plants Centre Research Institute, Turkish Ministry of Agriculture. Bread wheat cultivar Chinese Spring and wild emmer wheat lines TR39477 and TR38828 in Sabanci University (SU) were used in this research.

3.1.4. DNA Material

Flow sorted 5D chromosome short (5DS) and long arms (5DL) were obtained from J. Dolezˇel and his colleagues (IEB, Olomouc, Czech Republic; unpublished).

3.1.5. Equipments

Equipments used in this research are listed in Appendix C.

3.2. Methods: Barley microRNAs and Drought

3.2.1. Computer-based Identification of Barley microRNAs

3.2.1.1. Sequence Datasets

Reference miRNA dataset corresponded to a total of 1,988 mature miRNA sequences. It contained 1,763 mature miRNA sequences deposited in miRBase (version 13, March 2009, http://mirbase.org/) from 12 plants (Griffiths-Jones et al. 2008), as well as other miRNAs previously identified in close relatives of barley: 93 bread wheat and 132 *B. distachyon* miRNAs reported by Wei et al. and Unver and Budak, respectively (Unver and Budak 2009; Wei et al. 2009). *H. vulgare* expressed sequence tags (ESTs) were obtained from GenBank at the National Center for Biotechnology Information

(NCBI) (http://www.ncbi.nlm.nih.gov/, July, 2009) and corresponded to a total number of 525,527.

3.2.1.2. Homology-based *In Silico* microRNA Identification

Homology-based computational strategy developed by Zhang and his collegues was used for the identification of barley miRNAs (Zhang et al. 2005). This method is based on two important parameters: (1) homology of mature miRNA sequences to a previously known plant miRNA and (2) pre-established features of pre-miRNA secondary structure. A flow chart of this method is presented in Figure 5. Known plant mature miRNA sequences were searched as a query against *H. vulgare* ESTs using the Basic Local Alignment Search Tool (BLAST), nucleotide-nucleotide BLAST (BLASTN) algorithm, BLAST 2.2.21 (28 July, 2009) (Altschul et al. 1997) with parameters adjusted for short input sequences. EST sequences showing only 0–3 nucleotide mismatch(es) to a miRNA sequence query were retained and subjected to the Zuker folding algorithm for a second filtering. This was performed using the web-based software, MFOLD 3.2 (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi), which predicts lowest minimum folding free energy (MFE) secondary structure of a given sequence (Mathews et al. 1999; Zuker 2003). Generated outputs: predicted secondary structures represented as images, dot-bracket notations and connect files were downloaded along with the other characteristics of secondary structures. The minimal folding free energy index (MFEI), which is an important parameter to distinguish pre-miRNAs from other RNAs was calculated for each structure with the formula

$$MFEI=((MFE/\text{length of the RNA sequence})X100)/(G+C)\% \quad (1)$$

and further elimination was performed on the structures regarding to their consistency with pre-established pre-miRNA secondary structure characteristics to determine putative barley miRNAs (Yin et al. 2008; Zhang, Pan, and Anderson 2006; Zhang, Pan, Cobb, et al. 2006). Further validation for putative barley miRNAs was performed with the web-based tool RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), CLC RNA workbench (http://www.clcbio.com/index.php?id=1042), and MicroPC (μPC)

(http:// www.biotec.or.th/isl/micropc) (Mhuantong and Wichadakul 2009). Using μPC, homologs of putative barley miRNAs in other plants were also detected.

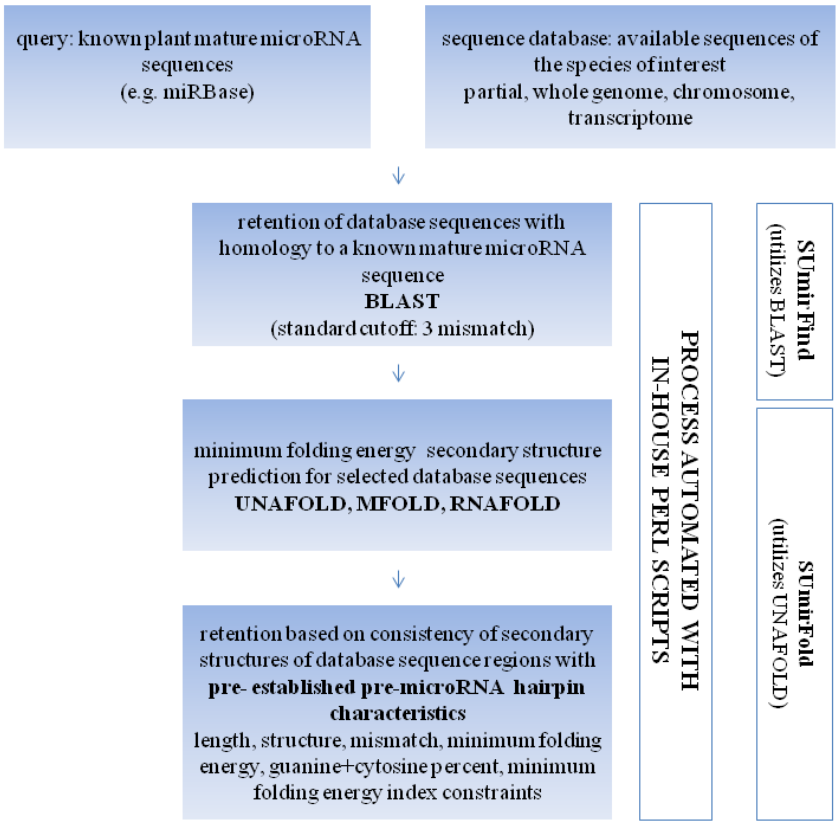

Figure 5 Flow chart showing steps of *in silico* homology based microRNA identification and its automation with two in-house perl scripts.

3.2.2. *In Silico* Identification of microRNA Targets

The target transcripts including complementary sequences of newly identified miRNAs were found by using the BLASTN algorithm. *H. vulgare* miRNAs targets detected were also subjected to homology search against the protein-coding mRNA databases of all plant species. Some criteria was previously applied to predict potential target transcripts of given plant miRNAs (Yin et al. 2008; Zhang, Wang, and Pan 2007; Zhang, Wang, Wang, et al. 2007). We have also followed previous principles which are (1) mismatches at complementary sites between miRNA and the target gene transcript should not be more than four nucleotides (Bartel, Lee, and Feinbaum 2004; Rhoades et

al. 2002; Schwab et al. 2005); (2) mismatches at positions 10 or 11 of the miRNA sequence at the site of complementarity between miRNA and target mRNA, which are assumed to be cleavage sites, are not allowed; however, up to three mismatches between 12–23 nucleotides can be allowed and (3) no more than three continuous mismatches are allowed in the miRNA–mRNA pair. miRU, a plant miRNA target finder software (http://bioinfo3.noble.org/miRNA/miRU.htm) was used for further validation as outlined by Unver and his collegues (Unver, Namuth-Covert, and Budak 2009).

3.2.3. Drought Responsive microRNAs and their Targets

3.2.3.1. Plant Materials, Growth Conditions and Dehydration Stress

Seeds of barley variant Bülbül-89, resistant to drought, were surface sterilized in 4% sodium hypochlorite for 10 min and pre-germinated in petri dishes with 5 ml milipore water for 11 days at 25◦C±1 at dark. Seedlings of a similar developmental stage were transferred to continuously aerated Hoagland's solution renewed every 3 days and grown under controlled conditions (15 hour photoperiod, temperature 23/21◦C, relative humidity 60/70%, and photon flux density of 600–700 µmolmeter^{-2}s^{-1}). At four-leaf stage, plant stress treatment was applied (Ergen et al. 2009). Plants were stressed by removing them from tanks and leaving on paper towels under the same lightning conditions for 8 hour while control plants were kept in fresh hydroponic solution. Root and leaf tissue samples from both stress and control plants were collected, directly frozen and stored at −80◦C.

3.2.3.2. Total RNA Isolation from Leaf and Root

Total RNA was isolated from leaves and roots of 8 hour stress and control samples as performed elsewhere (Ergen et al. 2009). Isolation was performed using 0.05 g frozen tissue using Trizol reagent (Invitrogen) according to manufacturer's instructions. The quality and quantity of isolated leaf RNAs were measured using a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE,

USA) and integrity of the isolated RNA was confirmed by separating the major ribosomal (rRNA) bands on agarose gels. DNase treatment of 1 µg of total RNA was performed in 10 µl reaction mixture with 1 U of RNase-free DNase I dioxyribonuclease I (Fermentas). The reaction was maintained at 37◦C for 30 min and terminated by adding 1 µl of 25 mM ethylenediaminetetraacetic acid (EDTA) followed by an incubation at 65◦C for 10 min. For miRNA quantification, DNase-treated samples were ethanol precipitated and then dissolved in 20 µl RNase-free water. All samples were stored at −20◦C.

3.2.3.3. Stem-loop Reverse Transcription of microRNAs

Stem-loop reverse transcription (RT) primers for predicted barley miR156, miR166, miR171 and miR408 were designed according to Varkonyi-Gasic et al. (Varkonyi-Gasic et al. 2007) (Appendix D). miRNA-specific stem-loop RT reactions were performed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) as outlined by Unver and his collegues (Unver et al. 2009). The miRNA stem-loop RT involved 1, 10, and 100 ng of total RNAs (1 µl), 1 µl 10 mM deoxynucleotide (dNTP) mix, 1 µl stem-loop RT primer (1 µM), and 7.75 µl nuclease-free water. They were incubated for 5 min at 65◦C and incubated on ice for 2 min. We added 2 µl first-strand buffer (10X), 4 µl 25 mM magnesium chloride ($MgCl_2$), 2 µl 0.1 M Dithiothreitol (DTT), 1 µl RNaseOUT (40 U/µl), and 0.25 µl SuperScript III (200 U/µl; Invitrogen) to each tube and performed the pulsed RT reaction as follows: 30 min at 16◦C, 60 cycles at 30◦C for 30 s, 42◦C for 30 s, and 50◦C for 1 s. The RT reactions were terminated at 85◦C for 5 min. With miRNA cDNA synthesis, we also performed no-RT primer and no-RNA control reactions.

3.2.3.4. microRNA Quantitative Real Time Assays

In order to experimentally validate barley miRNAs and measure their expression level differences in leaf and root tissues upon dehydration shock, quantitative real time polymerase qRT-PCR was performed. Reactions were performed using Brilliant II SYBR Green QPCR Master Mix (Stratagene) on an Icycler Multicolor Real-time PCR

Detection Systems (Bio-Rad) following the protocol in (Unver and Budak 2009). Using 3 µl RT stem-loop cDNA products qRT-PCR reactions were performed using 10 µl 2X master mix, 0.6 µl forward (300 nM), 0.6 µl reverse (300 nM) primers, 0.3 µl (30 nM) reference dye and 5.5 µl nuclease-free water. The specific forward primers were designed for each individual miRNA and a universal reverse primer was used (Varkonyi-Gasic et al. 2007) (Appendix D). Specified qRT-PCR thermal setup was adjusted as follows: heated to 95◦C for 10 min, followed by 40 cycles of 95◦C for 30 s, 56/58◦C for 1 min, and 72◦C for 30 s, followed by 72◦C for 10 min. The annealing temperature was optimized to 58◦C in miR408 quantification. The annealing temperature for the remaining miRNAs were optimized to 56◦C. The melting curves were generated using the following program: PCR products were denatured at 95◦C and cooled to 65°C. The fluorescence signals were collected continuously from 65◦C to 95◦C as the temperature increased at 0.2◦C per s. All reactions were repeated three times. Separation of PCR products was performed using 3% agarose gels.

3.2.3.5. Target mRNA Verification by Quantitative Real Time Assay

Expression level differences of computationally predicted barley miRNA target transcripts upon dehydration shock were measured with qRT-PCR experiments. These included miR156 target EST (AV910992.1), miR171 target EST (BQ461013.1) and miR408 target EST (BU995745.1). ESTs for target quantification analysis were selected based on two criteria: (1) protein found in BLAST search should be a previously published target of the related miRNA in another plant species and (2) The EST should have a possible protein-coding open reading frame (ORF) that can allow us to design qRT-PCR primers for the non-conserved 3′ untranslated region (UTR). The following criteria were set to design specific qRT-PCR primers for the above genes using Primer3: (1) Primer 3′ self complementary was set to 0; (2) primer annealing temperatures were limited to 62◦C 0±3 and (3) product size for primer pairs was limited to 80–120 base pair (bp). Total cDNAs were synthesized from 80 ng RNA using Superscript III First-Strand Synthesis System for qRT-PCR (Invitrogen) according to manufacturer's instructions. qRT-PCR analysis was performed as previously outlined (Unver and Budak 2009). Briefly, 1 µl of this cDNA was amplified with 300 nM of specific primers in a total of 20 µl volume using Brilliant II SYBR Green QPCR Master Mix

(Stratagene) with Icycler Multicolor Real-time PCR Detection Systems (Bio-Rad). The quantification was performed using 18s rRNA (GenBank ID:AF147501) as a normalizer and three independent PCR results with acceptable efficiency (1.8–2.2) were averaged. Specified qRT-PCR thermal setup was adjusted as follows: heated to 95◦C for 10 min, followed by 40 cycles of 95◦C for 30 s, 55◦C for 1 min followed by 72◦C for 7 min. The melting curves were generated using the following program: PCR products were denatured at 95◦C and cooled to 55◦C. The fluorescence signals were collected continuously from 55◦C to 95◦C as the temperature increased at 0.5◦C per s. All reactions were repeated three times. miRNA target and rRNA gene primers are listed in Appendix D.

3.2.3.6. Cleaved Target mRNA Identification by RNA Ligation Mediated Rapid Amplification of Complementary DNA ends

For the verification of computationally proposed miRNA targets and possible discovery of novel miRNA-cleaved mRNAs, we performed a modified version of 5′ RLM-RACE (Arenas-Huertero et al. 2009)(Wei et al. 2009). We applied RACE reactions using computationally proposed miRNA target ESTs: miR1436 target (GenBank ID: CB862673.1), miR156 target (GenBank ID: BE060620.2), miR408 target (GenBank ID: BU995745.1). For selection of EST targets of each miRNA for RACE analysis, first ESTs were *in silico* translated. Then they were searched against protein databases of *Viridiplantae* using nucleotide-protein BLAST algorithm (BLASTX) and protein-protein BLAST algorithm (BLASTP) for determination of the corresponding putative miRNA target transcripts. Selection of an EST for each miRNA was performed using the following criteria: (1) protein found in BLAST search should be a previously published target of the related miRNA in another plant species and (2) EST sequence should have an ORF encoding a protein similar in size to its protein homologues in other plant species. Specific reverse primers were designed for 5′ RLM RACE for each EST using Primer3 (Appendices D, E). Primers were designed at least 150–200 bp downstream of the miRNA cleavage site. The following criteria were set during primer design: (1) Primer 3′ self-complementary was set to zero; (2) primer annealing

temperatures were limited to 65◦C±5. (3) (G+C)% was set to 50±1; (4) primer size was set to 20–24. Control and stress-treated leaf total RNAs (1 μg) were ligated with adaptor and used as template for cDNA synthesis using the RLM-RACE kit (Ambion) following the manufacturer's procedures. To select mRNAs cleaved by miRNAs, calf alkaline phosphatase and tobacco acid pyrophosphatase steps were omitted. Using 5′ RACE outer primers (0.4 μM) and gene-specific reverse primers (0.4 μM) PCR amplification of cDNA fragments was performed. The PCR reaction and cycling conditions were setup following the manufacturer's protocol. Annealing temperatures were adjusted for the specific primers. PCR fragments were cloned and single clones were sequenced to identify the 5′ end of the amplified target genes. The corresponding barley ESTs for each cleaved sequence were found using the BLAST algorithm of GrainGenes against all GrainGenes sequences. (http://wheat.pw.usda.gov/GG2/blast.shtml) Then these ESTs were searched against all *Viridiplantae* protein-coding databases using NCBI BLASTX. All hits which had a score >30 were saved for further analysis. The cleaved sequences were also searched for their miRNA partners both among computationally identified miRNAs in this study and among 1,763 miRNAs deposited in miRBase. For the selection of miRNA partners from other species two criteria were followed: (1) No less than seven matches is allowed. (2) three mismatches are allowed because during computational prediction of new miRNAs, up to three mismatches with its homolog is accepted. Barley miRNAs were selected on the basis of no mismatch between miRNA and cleaved target complementary sites.

3.3. Methods: Wild Emmer Wheat microRNAs and Drought

3.3.1. microRNA Identification with Hybridization Chip

3.3.1.1. Plant Materials, Growth Conditions and Dehydration Stress

Wild emmer wheat [*T. turgidum* ssp. *dicoccoides* (Korn.) Thell.] drought-tolerant genotypes TR39477 and TR38828 were selected for the miRNA microarray study. Seeds were surface sterilized in 4% sodium hypochlorite for 10 min. They were vernalized and pre-germinated for 21 days at 4◦C in petri dishes with 5 ml milipore water at dark. They were transferred to hydroponics and grown under controlled conditions (16 hour photoperiod, temperature 24/22◦C, relative humidity 60%, and photon flux density of 600–700 μmolmeter^{-2}s^{-1}). Hydroponics in Hoagland's solution, application of shock dehydration stress, as well as collection and storage of tissues were performed according to methods outlined in (Ergen et al. 2009) and explained in detail in Section 3.2.3.2. Root and leaf tissue samples were collected from both stress and control plants at the fourth and eighth hour of stress.

3.3.1.2. Total RNA Isolation from Leaf and Root

Total RNA isolation from leaf and root tissues of 4 and 8 hour drought shocked and control plants was performed using Trizol reagent (Invitrogen) as outlined by (Ergen et al. 2009) and explained in detail in Section 3.2.3.2.

3.3.1.3. Microarray Chip Content and Hybridization to Arrays

The miRNA chip included 853 miRNA probes from 21 plant species corresponding to miRNA transcripts listed in miRBase (version 12, October 2008, http://www.mirbase.org/) (Griffiths-Jones et al. 2008) with 56 hybridization control probes. The miRNA microarray was synthesized in situ by LC Sciences (Houston, TX, USA; http://www.lcsciences.com/mirna.html) where chip hybridization was also performed. The microarray was probed with pooled RNA isolated from 4 *T. diccocoides* seedlings under each condition. 5 µg total RNA was used to probe the microarray. For each miRNA probe, 8 technical replicas were tested for the control condition and 4 replicas for each stress condition.

3.3.1.4. Microarray Data Analysis

An Axon Gene Pix 4000B Microarray Scanner was used for data collection and ArrayProTM image analysis software (Media Cybernetics, Silver Spring, MD, USA) was used for data extraction and image processing. The signal values were derived by background subtraction and normalization performed with a LOWESS method to remove system-related variations. A miRNA signal was accepted as detectable if it met two conditions: signal intensity higher than 3X background standard deviation, and spot coefficient of variation (CV) calculated with formula

$$CV = \text{signal standard deviation}/\text{signal intensity} \quad (2)$$

Lower than 0.5. Signals from four technical replicates each of RNA derived from stressed and control plants were compared using paired, two-tailed Student's *t* test; only signals with *P* values <0.05 and >3-fold increased or decreased differential expression were considered as significant. The clustering analysis was performed using Cluster 3.0 and heat map was visualized using Heatmap builder and TreeView (Eisen et al. 1998).

3.3.2. Additional Computational Analysis

3.3.2.1. Stem-loop Verification of microRNAs Identified by Chip

To ensure that the candidates are miRNAs rather than other sRNA types, their stem-loop forming pre-miRNA hairpins must also be detected. For this purpose, we searched all sequences corresponding to miRNA probes that gave a positive signal in the microarray study against *T. dicoccoides* ESTs allowing 3 mismatches. *T dicoccoides* ESTs were obtained from NCBI (http://www.ncbi.nlm.nih.gov) corresponding to a total of 9,343 sequences from biosamples LIBEST_023452, LIBEST_023453, LIBEST_023455 and LIBEST_023454 (Ergen and Budak 2009). MFE secondary structures were predicted for selected EST sequences, which were further evaluated regarding to their consistency with pre-established pre-miRNA secondary structure characteristics (Yin et al. 2008; Zhang, Pan, and Anderson 2006; Zhang, Pan, Cobb, et al. 2006), as noted in Section 3.2.1.2. Alignment and secondary structure prediction steps were performed using in house perl programs: two in-house Perl scripts: SUmirFind and SUmirFold, which will be explained in Section 3.4.1.2 (Lucas and Budak 2012).

3.3.2.2. Prediction of Targets for Drought Responsive microRNAs

Sequences of differentially expressed miRNA probes from the microarray study were used to interrogate sequences for target sites on the psRNAtarget web server (http://bioinfo3.noble.org/psRNATarget/), and updated version of miRU noted in Section 3.2.2 (Kantar et al. 2010; Unver et al. 2009). The analysis was first carried on *T. dicoccoides* sequences and than expanded to other *Triticum* species due to the limited availibility wild emmer wheat sequences. Target database used included *T. dicoccoides* ESTs from (Ergen and Budak 2009). *T. durum* unique transcripts from Plant Gene Database (PlantGDB) (http://www.plantgdb.org/). and *T. aestivum* gene index (release 9.0) (Lee et al. 2005).

3.4. Methods: Analysis of Bread Wheat 5D Chromosome microRNA Repertoire

3.4.1. Computer-based Identification of 5D Chromosome microRNAs

3.4.1.1.Sequence Datasets

A total of 5,940 mature miRNA sequences from 67 plant species deposited in miRBase were downloaded (version 19, August 2012, http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2011). In order to prepare a nonredundant query for miRNA prediction, in cases where multiple miRNAs had identical mature miRNA sequences, only one was retained, leaving a total of 3,228 unique, mature miRNA sequences. To be used for miRNA prediction, flow sorted *T. aestivum* (variant Chinese Spring) chromosome arms: 5DS and 5DL were sequenced using GS Titanium Rapid Library Preparation Kit, the GS FLX Titanium LV emulsion PCR (emPCR) Kit and GS FLX Titanium Sequencing (XLR70) Kit following the manufacturer's instructions (Roche). A total of 3,208,630 reads; 937,264 for 5DS and 2,271,366 for 5DL were obtained corresponding to 1.34x and 1.61x coverage, respectively (EBI Sequence Read Archive, accession number ERP002330, http:// www.ebi.ac.uk/ena/data/view/ERP002330) (Kurtoglu et al. 2013).

3.4.1.2. Automated Homology-based *In silico* microRNA Identification

For miRNA identification, the strategy first developed by Zhang and his collegues described in Section 3.2.1.2, was adopted (Zhang et al. 2005). However, the process was automated by using two in-house Perl scripts: SUmirFind and SUmirFold decribed in detail in our group's publications and represented in Figure 5 (Kantar et al. 2012; Kurtoglu et al. 2013, 2014; Lucas and Budak 2012). First, two separate databases were generated from sequence reads noted in Section 3.4.1.1 for 5DS and 5DL using the BLAST version 2.2.25+ (March 2011) (Camacho et al. 2009). Then, SUmirFind

program that uses BLAST version 2.2.25+ with parameters adjusted for short input sequences was run to search miRNA query referred in Section 3.4.1.1 against the generated databases. Cutoff set to 3, allowed the retention of database sequences, which had less than 3 base pairs of mismatch to a query miRNA sequence. Remaining sequences were then subjected to SUmirFold, utilizing UNAFold version 3,8 (an implementation of the Zuker folding algorithm) for prediction of their MFE secondary structures (Markham and Zuker 2008). SUmirFold initially predicts the secondary structure and examines base pairing between the putative miRNA:miRNA* duplex for all assembly sequences, remaining after SUmirFind implementation. For all hits passing this criteria, the sequence region surrounding the putative miRNA:miRNA* is excised, refolded and checked for several other pre-established characteristics of a pre-miRNA structure (Yin et al. 2008; Zhang, Pan, and Anderson 2006; Zhang, Pan, Cobb, et al. 2006) as noted in Section 3.2.1.2. Structures were further manually inspected to eliminate multi-branch loops and in cases where identical miRNAs were predicted from two similar query mature miRNA sequences, only one was retained. Results were evaluated both individually and collectively for 5DS and 5DL (Kurtoglu et al. 2013).

3.4.2. Additional Computational Analysis

3.4.2.1. Representation Analysis of Putativ e microRNA-coding Sequences

Representation for each miRNA was calculated by counting its corresponding stem-loop coding regions. In order to prevent overrepresentation, identical hits corresponding to the same miRNA were removed. Representation dataset included all identical pre-miRNA sequences predicted to be located in different reads or at different locations on the same read, in addition to all unique putative pre-miRNA sequences. Identical pre-miRNA sequences located at the same genomic region were also included separately to the fold analysis, in cases when the mature miRNA was located on the different arms of the hairpin. Representation was analyzed both individually and collectively for 5DS and 5DL (Kantar et al. 2012; Kurtoglu et al. 2013, 2014; Lucas and Budak 2012).

3.4.2.2. Repeat Analysis of Putative microRNA-coding Sequences

Repetitive elements in the pre-miRNA coding regions predicted to be present in the 5DS and 5DL were identified. For this purpose, pre-miRNA sequences were masked against a custom repeat library assembled from the *Triticeae* Repeat Sequence Database (TREP, release 10; http://wheat.pw.usda.gov/ITMI/Repeats/) using a semi-automated pipeline, RepeatMasker version 3.2.9 (www.repeatmasker.org). Transposable elements that are present in putative pre-miRNA coding regions in 5DS and 5DL were listed and compared (Hernandez et al. 2012; Kantar et al. 2012; Kurtoglu et al. 2013, 2014; Lucas and Budak 2012; Vitulo et al. 2011).

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3.4.2.3. *In silico* Target Identification of Putative microRNAs

First, known targets of homologous of predicted *T. aestivum* miRNAs were obtained from miRBase (version 19, August 2012, http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2011). Next, *T. aestivum* miRNA target prediction was also performed on a dataset including one mature miRNA sequence corresponding to each miRNA predicted, using the online software psRNAtarget against the DFCI Gene Index Release 12. (http://plantgrn.-noble.org/psRNATarget/) (Kantar et al. 2012; Kurtoglu et al. 2013, 2014). Possible target functions of newly identified miRNAs were searched manually using QuickGO (http://www.ebi.ac.uk/QuickGO/), a web based browser for gene ontology terms and annotations provided by the UniProt-GOA project at EBI.

3.4.2.4. *In silico* Expression Analysis of Putative microRNAs

To provide *in silico* pre-miRNA expression evidence, for each new miRNA detected in 5DS and 5DL, one corresponding pre-miRNA sequence was searched against the ESTs of *T. aestivum* in NCBI (January 2013, 1,286,372 sequences) using NCBI BLASTN. Hits above a threshold query coverage of 99% and maximum identity

of 98% were recorded for each potential miRNA. To identify candidate pre-miRNA coding ESTs, all EST matches were compared to the non-redundant protein database at NCBI using BLASTX. All ESTs matching any protein sequence at an e-value of 1E-03 or lower were considered to be protein-coding, and were eliminated. Predicted miRNAs and the corresponding accession codes of corresponding EST hits were listed (Kantar et al. 2012; Kurtoglu et al. 2013, 2014).

3.4.3. Mapping and Quantification of microRNA-coding Sequences

3.4.3.1. Plant Materials and Growth Conditions

T. aestivum (variant Chinese Spring), its nullitetrasomic and 5D deletion line series were grown. First, seeds were surface sterilized with 4% sodium hypochlorite for 10 min and vernalized in petri dishes with 5 ml milipore water for 3–4 days at 4^{o}C at dark. Seedlings were transferred to pots containing soil supplemented with 200 parts per million (ppm) nitrogren, 100 ppm phosphorus and 20 ppm sulfur and grown in normal greenhouse conditions (16 hour light at 22^{o}C and 8 hour dark at 18^{o}C). Leaf tissue was collected from adult plants (Chinese Spring, nullitetrasomic and deletion series) and stored at 80^{o}C. Two lines of the nulli-tetrasomic series (N5D-T5A and N5DT5B: with the genomic constitution of AABBAA and AABBBB, respectively) were used. These lines lacked homoeologous 5D chromosomes (nullisomic condition) that were replaced by another homoeologous chromosome pair (tetrasomic condition) : 5A and 5B in N5D-T5A and N5D-T5B, respectively. Four homozygous lines from the group-5 wheat chromosome deletion series (5DS-2, 5DS-5, 5DL-5, 5DL-7) with different deletion breakpoints were used. The length of the remaining chromosome arm in each deletion line is referred as the 'fraction length' (FL). Corresponding FL values of each deletion line used are given in Table 1.

Table 1 Fraction length values of homozygous bread wheat group-5 chromosome deletion lines used in this research

Deletion line	Fraction length value
5DS-2	0.78
5DS-5	0.67
5DL-5	0.76
5DL-7	0.29

3.4.3.2. Plant DNA and RNA Material

RNA isolation from frozen Chinese Spring leaf tissue was carried out using TRI Reagent (Sigma) according to the manufacturer's instructions as performed elsewhere (Ergen et al. 2009) (refer to Section 3.2.3.2 for RNA quality and quantity measurement, RNA integrity check and DNase treatment). First strand cDNA was synthesized from 100 ng of DNase treated RNA with RevertAid H- M-MuLV RT (Fermentas). Genomic DNA (gDNA) isolation from frozen leaf tissue of wheat (Chinese Spring, nullitetrasomic and deletion series) was performed using WizardH Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. All nucleic acid samples including flow sorted chromosome arms: 5DS and 5DL were stored at 20^{o}C.

3.4.3.3. Endpoint- and Reverse Transcription-PCR Screening of Preliminary microRNAs

To experimentally validate 5D chromosome localization of selected pre-miRNAs (miR169, miR5085, miR2118, miR5070, miR6220), PCR screening was carried out using DNA from flow sorted 5D chromosome arms: 5DS and 5DL. To identify 5D chromosome specific miRNAs, PCR screening of these pre-miRNAs using gDNA from Chinese Spring and nullitetrasomic lines that lack 5D chromosome (N5D-T5A and N5D-T5B) was also performed. Additionally, using gDNA from wheat group-5 deletion series, chromosome arm specific pre-miRNAs were screened to determine their location

on the chromosome arm. To check the expression of these pre-miRNAs in adult leaf tissue in wheat grown under standard conditions, Chinese Spring leaf cDNA was used for reverse transcription PCR (RT-PCR).

PCR reactions were performed using 1 µl (10 ng/µl) template (DNA or cDNA) and were performed in a 20 µl PCR mix including 2 µl 10X Thermus aquaticus (Taq) polymerase buffer (final concentration: 1X), 1.6 µl 2.5 mM dNTP (final concentration: 0.2 mM), 0.6 µl 10 µM primer mix (final concentration: 300 nM from each primer) and 0.1 µl of 5U/µl Taq polymerase (0.5 U) (Fermentas). 2.5 mM $MgCl_2$ (stock concentration: 25 mM) was used for the amplification of miR6220, miR5070 and miR2118 and this value was optimized to 2 mM and 3 mM for the miR5085 and miR169 amplicons. Thermal cycling setup was adjusted as follows: heated to 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 50°C/60.5°C/62°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min. For amplification of miR2118 and miR5070, the annealing temperature was optimized to 50°C and 60.5°C, respectively. The annealing temperatures for the remaining miRNAs were optimized to 62°C. Primers used for PCR analysis are listed in Appendix D. Separation of PCR products was performed using 3% agarose gels.

3.4.3.4. Quantitative Real Time PCR of Preliminary microRNAs

To quantify pre-miRNA gene copy number and expression in Chinese spring, qRT-PCR was performed using FastStart Universal SYBR Green Master (ROX) (Roche) on an Icycler Multicolor Real-time PCR Detection Systems (Bio-Rad). Using 1 µl of template (DNA or cDNA), qRT-PCR reactions were performed as 20 µl including 10 µl 2X Master mix and 0.6 µl primer mix (300 nM from each primer). Specified qRT-PCR thermal setup was adjusted as follows: heated to 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 56°C /58°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min. The annealing temperature was optimized to 56°C for miR6220 and miR2118 quantification. The annealing temperatures for the remaining miRNAs were optimized to 58°C. The melting curves were generated by collecting fluorescence signals from 55 °C to 95°C as the temperature increased 0.5°C with a dwell time of 10 s for 80 cycles. Pre-miR2118 gene copy number quantification could not be performed due to the

presence of an additional nonspecific band. For quantification of pre-miR5070, located both on 5D and other wheat chromosomes, additionaly analysis was performed using nullitetrasomic lines along with Chinese Spring to quantify its 5D specific gene copy number. Normalization was performed with BF474284 primers (Appendix D), located to the long arm of wheat chromosome 1A. For quantification, PCR efficiency calculations were performed using the program LinRegPCR retrieved from the publication of Rujiter and his colleagues (Ruijter et al. 2009).

3.5. Methods: Introgression of Drought-related Quantitative Trait Loci to Elite Cultivars

3.5.1. Plant Materials and Growth Conditions

Bread wheat varieties: South Australian cultivars: Kukri and RAC875 with differring physiological abilities to cope with osmotic stress (Izanloo et al. 2008); and public elite lines with high yield characteristics: Tosunbey and Bolal) were selected for the study. First, seeds were surface sterilized in 4% sodium hypochlorite for 10 min. They were vernalized and pre-germinated in petri dishes in 5 ml milipore water for 4-6 weeks at 4^{o}C at dark. Seedlings were transferred to pots containing soil supplemented with 200 ppm nitrogen, 100 ppm phosphorus, 25 ppm sulfur and 155 ppm potassium and grown in normal greenhouse conditions (refer to Section 3.4.3.1). Additionally, 100 ppm nitrogen was added to the soil at the 20th and 40th days of plant development. The sowing of plants was performed as multiple sets at 5-10 days intervals in order to render the simultaneous presence of different cultivars at the appropriate developmental stage for performing intercultivar crosses (Figure 6 a-b). Other methods were also used to synchronize development of different cultivars including keeping the faster developing cultivars at 10^{o}C to delay their growth and/or supplementing with additional fertilizers to increase the number of spikes.

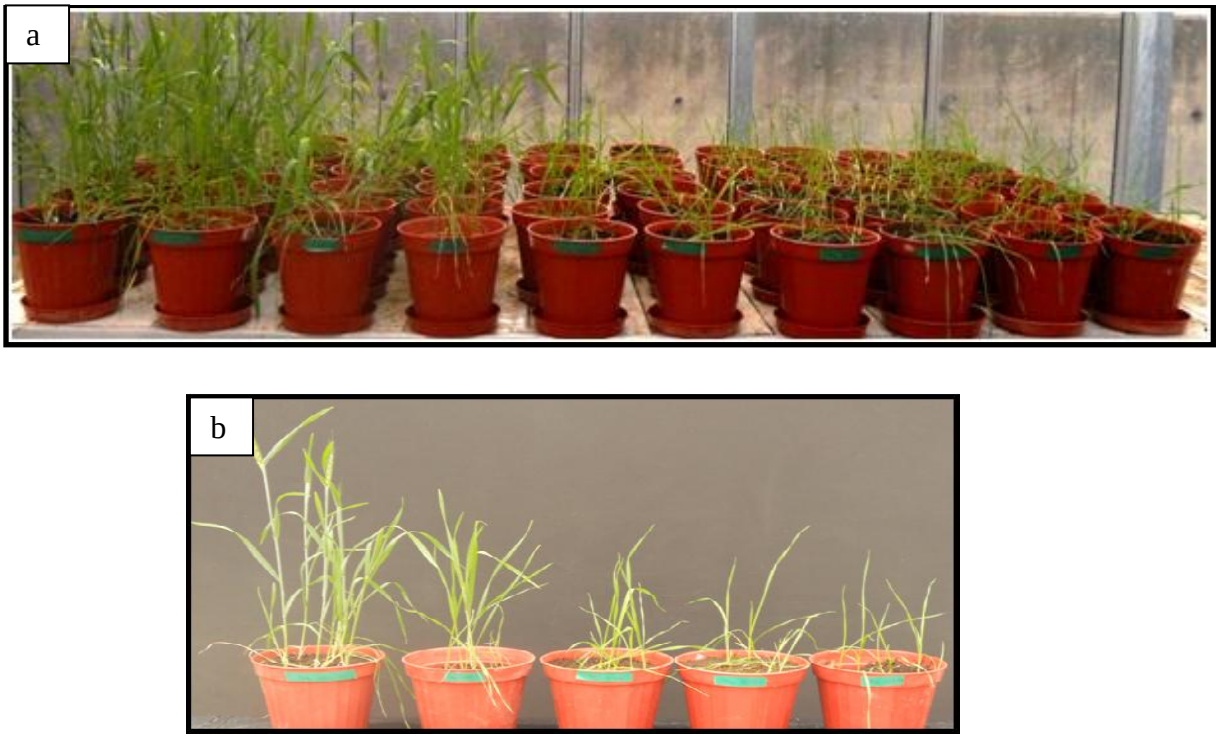

Figure 6 Multiple bread wheat plant sets grown for cross-pollination of different cultivars. (a) multiple plant sets sown with 5-10 days intervals (b) one representative Tosunbey plant from each set sown with 5-10 days intervals (photographed at 48 days after sowing of the first set)

3.5.2. Procedure for Intercultivar Crosses

Intercultivar crosses were performed between South Australian cultivars (Kukri, RAC875) and public elite lines (Tosunbey and Bolal) to produce new genotypes. In order to perform cross-pollination between two distinct varieties, two spikes, each from a plant of different genotype (one Australian cultivar and one elite line) were selected. One of these spikes were prepared as the female parent and the other as the male parent. During wheat development, heading is followed by flowering, in which pollination and fertilization occur. Selection of the female and male spikes were performed in the transition stage from heading to flowering.

Wheat spike contains several spikelets, and a spikelet has multiple florets with male and female reproductive organs. Each floret has one pistil containing stigma and

ovary; and three anthers that contain pollens. Maturity begins at the central spikelet of the head and spreads outwards. Spikes were selected to be used as a female if their heads were still partially encapsulated by the flag leaf sheath and anthers in their central spikelet were still green (Figure 7 a). At the same time, the availibility of spikes to be used as males during cross-pollination was also checked and any spike with its base leveling with the base of the flag leaf; slightly more mature than the female and would soon begin to produce pollen were spotted and marked as prospective males.

In order to prepare the female spikes for cross-pollination, basal and apical spikelets, and inner florets of the remaining spikelets were eliminated with a fine point forceps. Then using an embroidery scissors, the top half of every spikelet was cut, without injuring the pistil and the spikes were emasculated by the removal of all the anthers with the forceps (Figure 7 b). The vulnearable female spikes were covered with a glassine crossing bag to protect them from damage and polen (Figure 7 c).

A few days later after the preparation of the female (approximately three days), when the females and males were ready, cross-pollinations were performed. At this stage, the females were sexually mature: their florets were opened and stigmas were largened. The base of the males spikes were higher than the base of the flag leaf and in these males flowering was either in process in the central spikelets or about to begin (Figure 7 a). With the scissors, the top of the male spikes were cut and males were stimulated by rubbing and exposure to sunlight (Figure 7 b). Cross-pollination was performed by the transfer of suitable anthers, which have not lost their pollens to self-fertilization, to the female spikelets. The cross-pollinated females were reclosed with a bag (Placido et al. 2013; Randhawa et al. 2009) (Figure 7 c). After appropriate time, Filial 1 (F1) seeds were collected from cross-pollinated females.

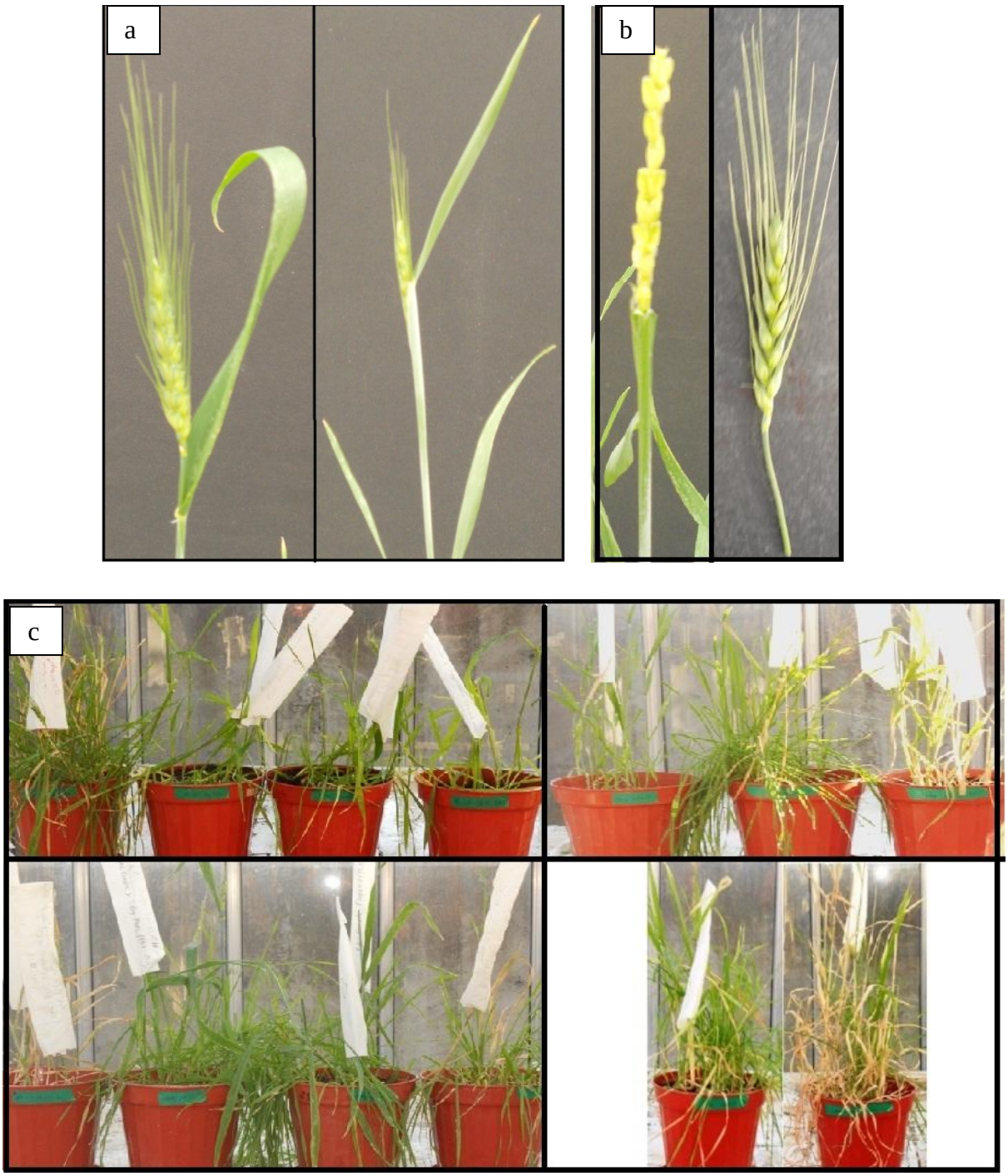

Figure 7 Procedure of cross-pollination. (a) spikes at the appropriate developmental stage to be used as a male (left) and female (right) (b) female (left) and male (right) spikes prepared for crossing (c) female plants for cross-pollination: Kukri (upper left), Tosunbey (upper right), RAC875 (lower left) and Bolal (lower right)

3.5.3. Molecular Screening for Introgression

One of the drought related QTLs previously identified through assessment of DH populations derived from crosses between RAC875 and Kukri, in field trials under water limited conditions, was selected for the study (Bennett, Izanloo, Edwards, et al. 2012; Bennett, Izanloo, Reynolds, et al. 2012; Bennett, Reynolds, et al. 2012). In order to screen plants for the presence of this locus mapped to bread wheat chromosome 3B, a linked polymorphic SSR: XBARC77 was used as a molecular marker (Somers, Isaac, and Edwards 2004) (Figure 8). Primers to perfom PCR amplifications of XBARC77 were obtained from GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and are listed in Appendix D.

PCR screening of drought related QTL was performed in parentals, F1, BC1F2, BC2F2 and BC3F2 plants (refer to Sections 3.5.2 and 3.5.4). Leaf tissue from plants were collected, directly frozen and stored at −80◦C. DNA Isolation was carried out with WizardH Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. PCR mix included 2 µl (50 ng) DNA, 1.6 µl (25 mM) $MgCl_2$, 1.6 µl (2.5 mM) dNTP, 0.25 µl (10 µM) forward and reverse primers and 0.1 µl (5U/µl) Taq polymerase (Fermentas). PCR temperature cycling conditions was set to 95°C for 5 min, 35 cycles of 95°C for 1 min, 60°C for 30 s, 72°C for 30 s, and 72°C for 10 min. Separation of PCR products was performed using 2.3% agarose gels.

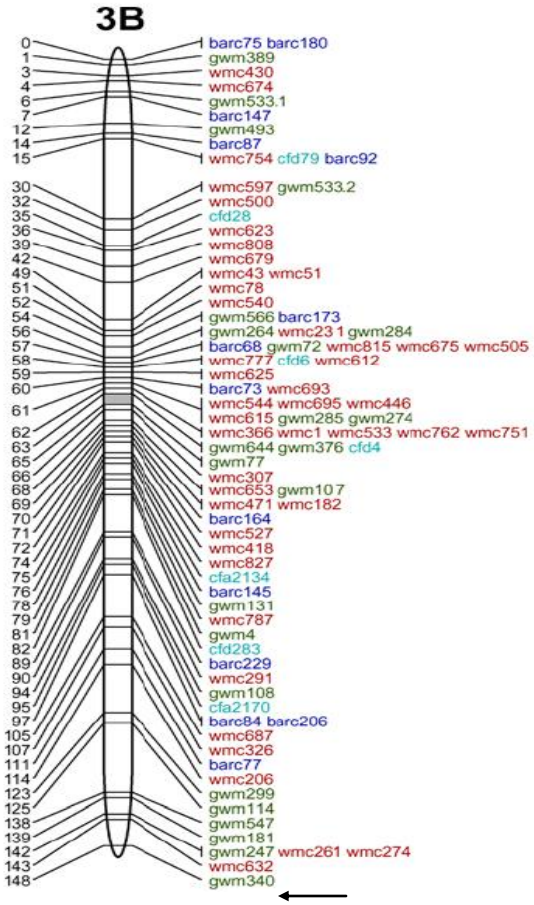

Figure 8 Drought related quantitative trait loci mapped to bread wheat chromosome 3B using Kukri and RAC875 recombinant inbred lines.

3.5.4. Backcrossing Against the Parental Background

F1 seeds were sown and grown using soil and greenhouse conditions noted in Section 3.5.1. After plant self-pollination and maturation, F2 seeds were collected. In order to limit the loss of good yield characteristics from the elite cultivar, backcrossing against parental genotypes was performed until BC3F2. In each backcrossing trial, plants were grown in soil and greenhouse conditions noted in Section 3.5.1. Parentals were sown as multiple sets at 5-10 days intervals. Cross-pollination was performed following the procedure in Section 3.5.2. Prior to further backcrossing, in each step, the obtained plants (F1, BC1F2, BC2F2 and BC3F2) were screened with methods outlined in Section 3.5.3 for the presence of the drought related QTL.

4.

4.RESULTS

4.1. Barley microRNAs and Drought

4.1.1. Putative Barley microRNAs and their Characteristics

Using *in silico* homology-based method, a total of 28 putative barley miRNAs were identified (Table 2, Appendix F). Of the identified *H. vulgare* mature miRNAs, 15 (52%) were found to be located in the 3′ arm, while the rest (48%) were in the 5′ arm of the stem-loop pre-miRNA hairpin. Lengths of barley mature miRNA sequences were observed to vary between 20 and 24 nucleotides. Most of the barley mature miRNA sequences (18 of the 28) had uracil as their first nucleotide in agreement with other results (Zhang, Wang, and Pan 2007)(Zhang, Wang, Wang, et al. 2007). Predicted barley miRNAs were classified into 18 miRNA families. The family of miR-172 was found to contain four members, while four other families (miR-160, miR-166, and miR-168) included two members and the remaining miRNA families were represented by only one member (Table 2).

Through μPC searches, possible homologues in other plants were identified for all putative barley mature miRNAs with the exception of miR1119, miR1139, and

miR2055. Most of the barley miRNA homologues were detected in bread wheat, maize and tomato (Appendix G). Conservation across other plant species was further analyzed for putative barley miR160a pre-miRNA. Across plant species, miRNA sequence of miR160a was found to be more conserved than its corresponding miRNA* sequence (Figure 9).

Pre-miRNA sequences of *H. vulgare* were observed to vary both in structure and in size. The size of identified pre-miRNAs ranged between 46–114 nucleotides in length with an average sequence size of 77.14±18.48 and median of 78 (Table 3). Eighteen of the detected pre-miRNAs contained more than 70 nucleotides in agreement with previous results (Unver and Budak 2009; Yin et al. 2008) (Table 2). MFE is used for the determination of RNA and DNA structures and lower MFE provides higher stability for secondary stem-loop hairpin structure. Previous studies had shown that pre-miRNAs have negative MFE and many researchers noted that their predicted pre-miRNAs have negative lower MFE value. MFEs found for the putative barley miRNAs here varied from −14.7 to −63.6 kcal/mol (with an average of −30.88±13.71 and median of −27.4 kcal/mol; Tables 2 and 3) representing good agreement with previous plant miRNA identification studies (Yin et al. 2008; Zhang, Pan, and Stellwag 2008; Zhang, Wang, and Pan 2007; Zhang, Wang, Wang, et al. 2007). MFEI incorporates major characteristics for pre-miRNAs including MFE of secondary structure, as well as sequence length and percent of guanine+cytosine ((G+C)%) content. Therefore the index provides a valuable criteria to distinguish miRNAs from other types of RNAs. MFEIs of other RNA types were characterized: transfer RNAs (tRNAs) (0.64), rRNAs (0.59), and mRNAs (0.62–0.66) and miRNAs were observed to have higher MFEIs than other types of RNAs (Schwab et al. 2005; Yin et al. 2008). In consistency with previous studies, here, MFEIs of predicted barley pre-miRNAs were detected to be higher (average 0.805±0.269 with median of 0.79) than other RNA types. For the majority of putative barley pre-miRNAs, this index was found to be greater than 0.66 (Tables 2 and 3).

Table 2 List of computer based newly identified barley microRNAs and their characteristics

New barley microRNA	Barley mature microRNA sequence	Accession	Homologous microRNA	Location	ΔG (kcal/mol)	LP	LM	G+C (%)	MFEI
miR1119	uggcacggcgcgaugcucagucag	GH227471.1	tae-miR1119	3'	-22.9	86	24	58.2	0.45
miR1120	acauucuuauauuaugggacggag	BJ486588.1	tae-miR1120	3'	-42.6	84	24	35.7	1.42
miR1121	cauagugaucuaaacgcucuua	FD522234.1	tae-miR1121	3'	-28.3	57	21	50.3	0.99
miR1122	uagauacauccguaucuaaa	EX593109.1	tae-miR1122	5'	-25.8	90	21	32.3	0.89
miR1126	ucaacuauggacuacauacggaa	BU974512.1	tae-miR1126	5'	-30.2	104	23	31.8	0.91
miR1139	gaaguaacuuagacuaguaaca	BQ471108.1	bdi-miR1139	5'	-23.6	46	21	34.8	1.47
miR1436	acauuaugggacggagggagu	EX572464.1	osa-miR1436	3'	-29.4	63	21	44.5	1.05
miR1438	uuuuggaacggagugaguaau	GH215029.1	osa-miR1438	3'	-37	91	21	34.1	1.19
miR156a	ugacacgagagagagaagac	FD528446.1	sbi-miR156a	5'	-18	75	21	56	0.43
miR159a	uuuggauugaagggagcucug	BJ456281.1	tae-miR159a	3'	-26.5	88	21	50	0.61
miR160a	ugccuggcucccuguaugccu	BF622299.2	tae-miR160a	3'	-39.7	100	21	51	0.78
miR160b	ugccuggcucccuguaugccu	BU986263.1	tae-miR160b	5'	-21.6	65	21	55.4	0.6
miR165	ucggaccaggcuucauucccc	BQ760548.1	tae-miR165	3'	-51.5	107	21	60.8	0.8
miR166m	ucggaccaggcuucauucccc	BQ760548.1	osa-miR166m	3'	-40.2	78	21	64	0.8
miR166n	ucggaccaggcuucauucccc	BY841849.1	osa-miR166n	3'	-63.6	102	21	59.9	1.04
miR168a	ucgcuuggugcagaucgggac	CA003609.1	osa-miR168a	5'	-51.6	86	21	73.3	0.82
miR168b	ucgcuuggugcagaucgggac	CA003589.1	tae-miR168b	5'	-42.4	66	21	69.9	0.96
miR171	ugauugagccgugccaauauc	CA009309.1	tae-miR171	5'	-44.5	78	21	61.5	0.93

miR172a	aggaucuugaugaugcugcc	GH215809.1	tae-miR172a	5'	-16	53	20	43.4	0.69
miR172b	agaauccugaugaugcugcag	GH213313.1	tae-miR172b	5'	-16	59	21	42.4	0.64
miR172c	gaggaucuugaugaugcugcc	BY867399.1	tae-miR172c	5'	-15.3	52	21	44.3	0.65
miR172d	aggaucuugaugaugcugccg	CX627187.1	tae-miR172d	5'	-15.3	52	21	46.2	0.64
miR2055	uuuccuugggaagggggguuuu	BG416283.1	osa-miR2055	3'	-14.7	63	21	30.2	0.77
miR395a	uccaaagggaucgcauugucu	GH212603.1	tae-miR395a	3'	-21.6	67	21	58.2	0.54
miR397	guugagugcagcguugaugaa	CA014459.1	tae-miR397	5'	-53.9	85	21	69.5	0.91
miR399g	ugccaaaggagauuugccccg	AV835204.1	vvi-miR399g	3'	-17	78	21	48.8	0.44
miR408	cugcacugccucuucccugcg	GH218076.1	osa-miR408	3'	-22.8	71	21	67.7	0.51
miR444	uugcugccucaagcuugcugc	CB882722.1	bdi-miR444	3'	-32.7	114	21	46.5	0.62

Accession: NCBI Genbank ID of the expressed sequence tag used for microRNA prediction (http://www.ncbi.nlm.nih.gov/, July, 2009), Homologous microRNA: miRBase ID of the query microRNA used for microRNA prediction (version 13, March 2009, http://mirbase.org), Location: location of the mature microRNA on the preliminary microRNA hairpin, ΔG: minimum folding free energy of the microRNA hairpin, LP: length of preliminary microRNA, LM: length of mature microRNA, G+C: guanine+cytosine percent of preliminary microRNA sequence, MFEI: minimum folding free energy index, tae: *Triticum aestivum*, bdi: *Brachypodium distachyon*, osa: *Oryza sativa*, sbi: *Sorghum bicolor*, vvi: *Vitis vinifera*

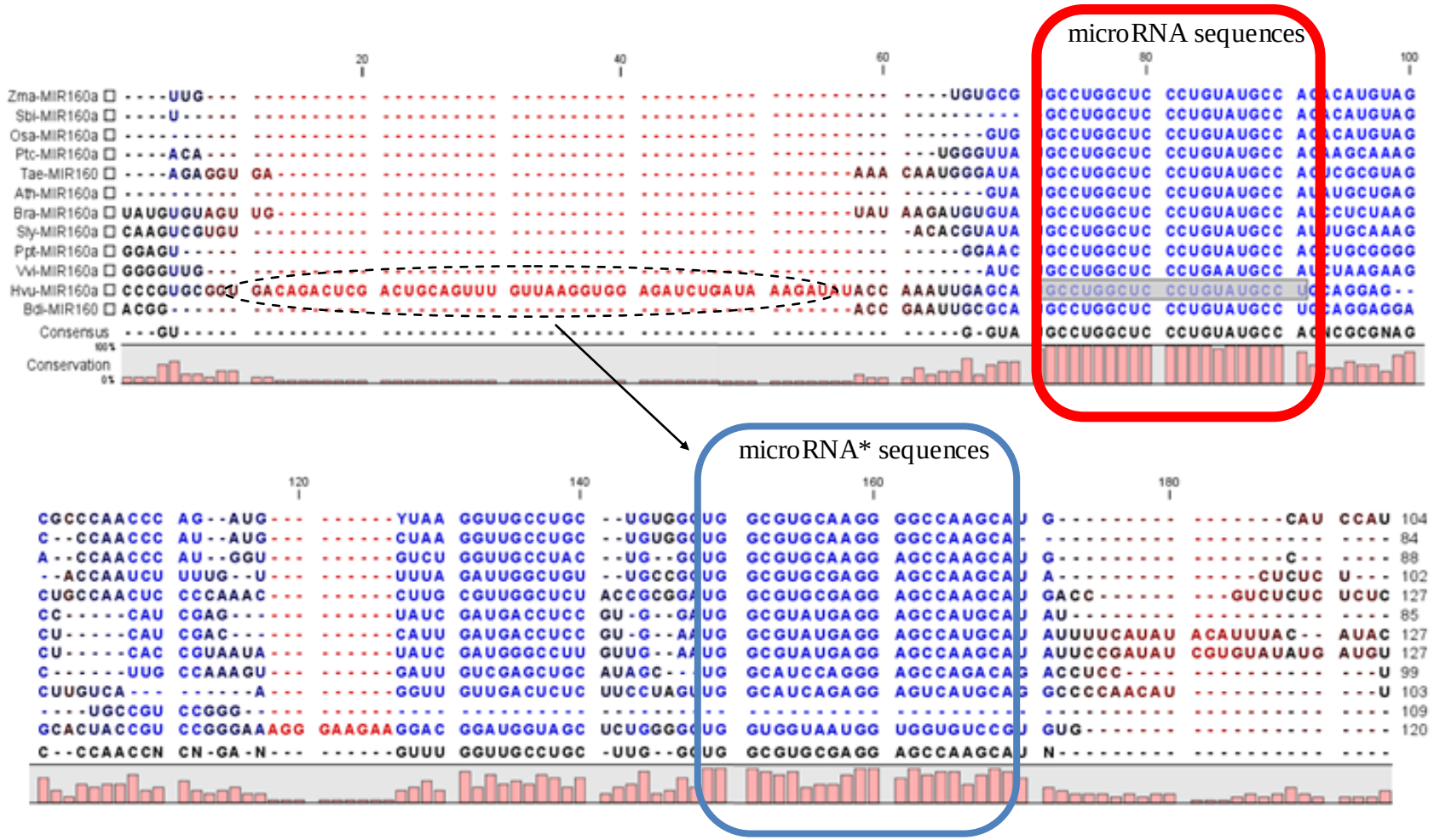

Figure 9 Multiple sequence alignment of predicted barley preliminary microRNA160a and preliminary microRNA160a's in other species. (miRBase version 13, March 2009, http://mirbase.org)

Table 3 Major characteristics of predicted barley preliminary microRNAs

Characteristic	Minimum	Maximum	Median	Average	Standard deviation
Sequence length	46	114	78	77	18
G+C (%)	30.2	73.3	50.2	50.8	12.6
MFEI	0.43	1.47	0.79	0.81	0.27
ΔG (kcal/mol)	-63.6	-14.7	-27.4	-30.9	13.7

G+C: guanine+cytosine percent of sequence, MFEI: minimum folding free energy index, ΔG: minimum folding free energy

4.1.2. Putative Barley microRNA Targets

Regulation of gene expression at the post-transcriptional level is provided by binding of miRNAs to complementary sites in target mRNA. By the guidance of miRNA the target

transcript is cleaved or its translation is inhibited (Bartel et al. 2004). We predicted putative mRNA targets of predicted barley miRNAs by searching their mature miRNA sequences against *H. vulgare* EST database and further filtering based on additional criteria (refer to Section 3.2.2). We found a total of 445 potential barley miRNA targets and at least one target was identified for each barley miRNA query except miR166n, miR166m, miR168a, miR168b, miR160b, and miR399g (Appendix H). Barley miRNA target genes were observed to vary in sequence and function, and most of them were classified as transcription factors, as well as functional proteins in plant metabolism, and stress response in consistency with other studies (Bonnet et al. 2004; Rhoades et al. 2002; Zhang, Pan, and Anderson 2006).

4.1.3. Dehydration Responsive Barley microRNAs

In order to detect whether some of the predicted barley miRNAs were regulated by drought, we performed qRT-PCR for quantification of selected barley miRNAs. Four of the 28 predicted miRNAs were measured in leaf and root tissues under dehydration stress conditions. Induced barley miRNAs in leaf tissue were: miR156, miR166, miR171 and miR408. In root tissue, expression of miR156, miR171, and miR408 were not changed upon dehydration stress, while miR166 was found to be suppressed (Figure

10). The qRT-PCR products were shown to be specific by melting analysis in qRT-PCR and agarose gel electrophoresis along with no-RT and no-RNA controls (Figure 11).

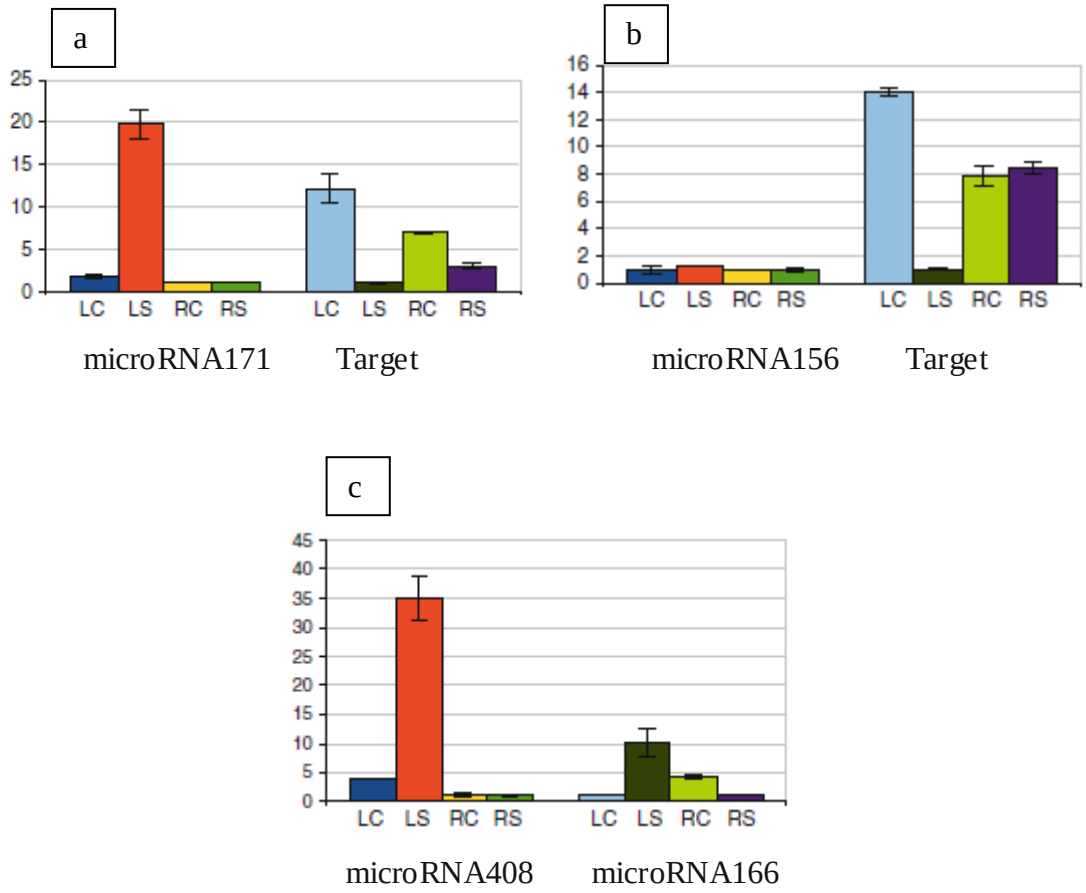

Figure 10 Quantification of barley microRNAs and their targets in leaf and root tissues in response to dehydration. (a) microRNA171-target BQ4610131.1 (b) microRNA156-target AV910992.1 (c) microRNA408-microRNA166
LC: leaf control, LS: leaf stress, RC: root control, RS: root stress
Quantification is based on quantitative real time PCR results and designated with bar graphs.

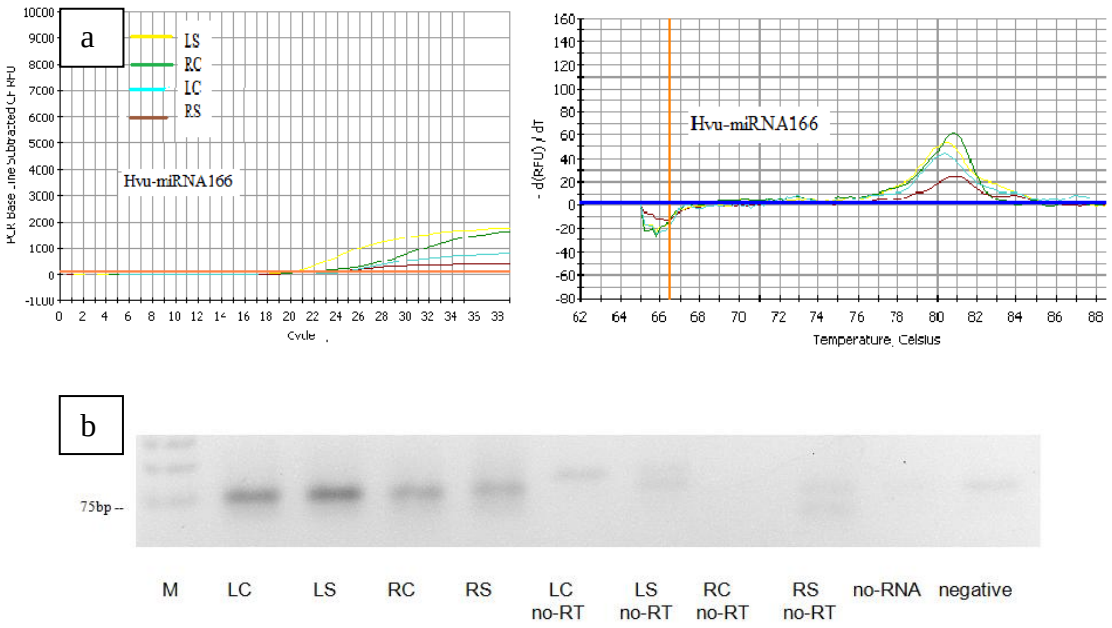

Figure 11 Barley microRNA166 amplification in control and drought stressed leaf and root tissues and confirmation of specific amplification with no reverse transcription and no RNA controls through melting curve analysis and agarose gel electrophoresis (a) amplification and
melting curves of quantitative real time PCR (b) agarose gel electrophoresis of quantitative real time PCR products
LC: control leaf, LS: drought stressed leaf, RC: control root, RS: drought stressed root, bp: base pairs, M: RNA ladder, no-RT: no reverse transcription control, no-RNA: no RNA control, negative: negative control

4.1.4. Dehydration Responsive Barley microRNA Targets

One *in silico* identified target for each miRNA quantified was selected for qRT-PCR, except for miR166, for which no target could be predicted. Expression levels of the target genes of barley miR156, miR171, and miR408 were quantified relative to controls in dehydration shocked barley root and leaf tissues. miR156 and miR171 target mRNA levels were found to be suppressed in stress applied leaf samples. Since an inverse correlation between levels of miR156, miR171 expression, and their corresponding target mRNA transcripts was observed, drought triggered regulation of mRNA expression by these miRNAs in barley leaf was validated. However, in dehydration-stress-treated root samples, Hvu-miR156 target was seen to be increased, while miR171 and miR408 targets were decreased. These changes in target transcripts

can be due to other mechanisms unrelated to miRNA-guided regulation (Figure 10). miR408 expression was not detected in either control or stressed leaf samples.

4.1.5. Drought Induced Target Cleavage by Barley microRNAs

Experimental validation of target mRNAs with 5′ RLM-RACE takes advantage of the preferred mode of action of plant miRNAs, which is target cleavage. In this work a modified 5′ RLM-RACE experiments were performed with total RNA extracted from leaf stress tissues. The predicted target ESTs of miR156, miR408, and miR1436 could not be verified with 5′ RLM-RACE. On the other hand, we have detected seven new target transcripts cleaved by miRNA-guided RISC cleavage (Appendix I). The cleaved sequences match with 13 ESTs and these ESTs were found to encode 15 protein homologues (Table 4). These sequences are most likely to be targets of six computationally proposed *H. vulgare* miRNAs: miR165, miR166, miR156, miR2055, miR171, miR172, miR397 and miR159. It is also possible that barley homologues of miRNAs in other species might target for cleavage. Possibly these transcripts are the targets of miR408, miR1510, miR1511, miR5p, miR538, miR1214, and miR853 (Appendix J).

Table 4 Expressed sequence tag and protein hits obtained by computational analysis of barley cleaved sequences identified using RNA ligation mediated rapid amplification of complementary DNA ends

Sequence	Expressed sequence tag ID	Protein ID
1.	CA021754	ref|XP_002532325, ref|NP_195851.2, emb|CAB85535.1
2.	BE421101	gb|ABR26072.1, emb|CAL55828.1, emb|CAJ43715.1, emb|CAJ43714.1
3.	CB014764, CB019633	gb|ABA27052
4.	CV0547724,CV054294	gb|ABA27052
5.	BF263515	gb|ABC74511.1,gb|ABC74510.1, gb|ABC74512.1
6.	Bcl1163ct1723cn5537, BF619398, barley1_14852	gb|ABA974982, ref|NP_566111.1, gb|ABG21980.1, ref|XP_002523062.1,gb|AA027294.1,emb|CAA12242.1, ref|NP_001148843.1
7.	BF617393, BE437840, BE437720	prf|1603356c, dbj|BAB85481.1, ref|YP_784093.1, dbj|BAC57648.1, emb|CA002552.1
Sequence	**Protein**	**Protein family**
1.	ubiquitin ligase	zinc finger in N-recognin (UBR box) domain
2.	biosynthesis protein, H+-ATPase, sinapyl alcohol dehydrogenase, 4-coumaryl-CoA ligase	alcohol dehydrogenase GroES-like protein, zinc-binding dehydrogenase
3.	TO71-3	-
4.	TO71-3	-
5.	DRF-like transcription factor DRFL2	AP2 domain found in APETALA2 and EREBP
6.	transducin family protein, WD repeat protein, RGA-like protein, proline rich protein	WD40 domain, DELLA protein N terminal domain, GRAS family transcription factor, hydrophobic protein from soybean (HPS) like family
7.	trnA, ACRS protein, resistance complex I2C-1, ATP synthase CF0 subunit 1	p-loop NTPase

Sequence: cleaved sequence identified by RNA ligation mediated rapid amplification of complementary DNA ends, Expressed sequence tag ID: Graingenes barley expressed sequence tag(s) that the cleaved sequence gives hit to (http://wheat.pw.usda.gov/GG2), Protein ID: homologue NCBI *Viridiplantae* protein(s) that the expressed sequence(s) gives hit to above a score of 30

4.2. Wild Emmer Wheat microRNAs and Drought

4.2.1. Wild Emmer Wheat Root and Leaf microRNAs

A plant miRNA chip microarray was used to detect differentially regulated miRNAs at 4 and 8 hour drought shock conditions in leaf and root tissues of *T. diccocoides*. Dehydration shock was selected as the stress condition because, as reported by (Ergen et al. 2009), for profiling the majority of drought-responsive genes. A total of 205 miRNAs (out of 853 miRNA probes on chip) were detected in unstressed tissues. Of these, 46 were found to be leaf specific and 21 were found to be root specific, the remaining 138 miRNAs being detected in both leaf and root tissues (Appendix K). In drought-stressed tissues, a total of 438 miRNAs (out of 853 miRNA probes on chip) were detected. Of these, 78 were specific to 4 hour stressed leaves; 43 were specific to 8 hour stressed leaves; and 20 were leaf specific and detected after both stress periods. Among root-specific miRNAs, 48 were specific to 4 hour stressed roots, 7 were detected only after 8 hour stress, and 15 were found to be present at both time points. Expression of 19 miRNAs was detected in both leaf and root tissues only at 4 hour of dehydration, and 8 miRNAs in both tissues only after 8 hour of drought stress. Finally, 143 miRNAs were expressed in both tissues under all drought treatments, the majority (135) of which had also been detected in also in unstressed tissues (Appendix L).

4.2.2. Dehydration Responsive Wild Emmer Wheat microRNAs

The microarray results showed that the expression of 5 of the 205 miRNAs expressed in control tissues was not detected during drought stress, whereas 236 out of 438 miRNAs expressed in stressed tissues were not detected in control tissues. This indicates that drought stress causes an overall increase in the number of miRNAs expressed. Differential expression due to drought stress was analyzed for those 141 miRNAs that were detected both in controls and stress conditions (Appendices K, L). miRNAs that showed >3-fold increased or decreased expression with a P value <0.05 were determined (Table 5). In leaf tissue, miR1867 and miR896 were found to be

differentially regulated under both drought conditions; miR1867 was upregulated at both timepoints while miR896 expression was downregulated after 4 hour but upregulated after 8 hour stress. Also after 8 hour stress, probes for 2 different miR398 family members were upregulated while miR528 was downregulated. In root tissue, miR474 was strongly induced under both drought treatments, similary at both stress time points, miR398 was also upregulated. After 4 hours of drought, levels of miR396 transiently decreased while miR1450 increased, but both had returned to control levels by 8 hour post-stress induction. Roots that had been drought-stressed for 8 hour showed the most changes in miRNA levels: in addition to miR474 and miR398, four other miRNAs (miR156, miR894, miR1432 and miR1881) were induced, while miR166 and miR171 were downregulated. The distribution of differentially expressed miRNAs by tissue and drought condition is displayed by the venn diagram (Figure 12).

Table 5 Wild emmer wheat microRNAs complementary to microarray probes showing altered expression in response to drought stress

Tissue-drought stress condition	microRNA	Homologous microRNA probe	Signal intensity		Mean fold change from control to stress	P value
			Control	Stress		
Leaf-4 hour	miR1867	osa-miR1867	32.1 ± 1.8	116.9 ± 10.9	+3.89	0.0004
	miR896	ppt-miR896	4,060 ± 423	1,252 ± 18.8	-3.25	0.0009
Leaf-8 hour	miR1867	osa-miR1867	30.4 ± 8.8	151.6 ± 39.6	+4.87	0.0071
	miR398	pta-miR398	8.23 ± 3.31	61.5 ± 8.9	+8.19	0.0005
		ath-miR398b	25.84 ± 5.55	96.0 ± 6.8	+3.98	<0.0001
	miR528	osa-miR528	1,985 ± 413	459.9 ± 63.1	-4.26	0.0045
	miR896	ppt-miR896	1,552 ± 154	5,290 ± 161	+3.36	<0.0001
Root-4 hour	miR1450	ptc-miR1450	21,538 ± 1,536	85,708 ± 3,065	+3.99	<0.0001
	miR396	osa-miR396d	2,379 ± 462	790.6 ± 69.9	-3.02	0.0056
	miR398	ath-miR398b	9.49 ± 2.29	43.96 ± 7.03	+5.11	0.0012
	miR474	ptc-miR474c	36.0 ± 10.0	1,940 ± 347	+54.93	0.0016
		ptc-miR474a	50.1 ± 15.8	2,164 ± 287	+46.40	0.0007
		ptc-miR474b	46.1 ± 8.7	2,067 ± 331	+44.62	0.0012
Root-8 hour	miR1432	osa-miR1432	73.7 ± 9.2	215.1 ± 21.3	+3.17	0.0002
	miR156	ptc-miR156k	291.8 ± 60.2	1,255 ± 73.2	+4.51	<0.0001
		vvi-miR156e	76.6 ± 15.7	232.9 ± 31.4	+3.15	0.0006
	miR166	vvi-miR166a	118.8 ± 31.3	32.8 ± 10.3	-4.48	0.0083
		osa-miR166k	343.5 ± 88.2	104.1 ± 18.5	-3.19	0.0105
		ptc-miR166p	439.1 ± 74.6	146.4 ± 26.0	-3.10	0.0023
	miR171	osa-miR171h	161.2 ± 25.1	52.99 ± 4.38	-2.91	0.0027
	miR1881	osa-miR1881	20.5 ± 1.4	281.9 ± 24.4	+13.87	0.0002
	miR398	ath-miR398b	8.67 ± 1.89	66.3 ± 11.6	+7.70	0.0018
		ath-miR398a	7.97 ± 4.43	61.8 ± 13.4	+7.64	0.0023
		pta-miR398	7.18 ± 3.21	42.5 ± 7.4	+5.13	0.0009
	miR474	ptc-miR474b	380.4 ± 70.0	9,568 ± 387	+26.12	<0.0001
		ptc-miR474c	361.2 ± 58.7	9,375 ± 587	+25.90	0.0001
		ptc-miR474a	394.7 ± 73.8	9,433 ± 430	+25.02	<0.0001
	miR894	ppt-miR894	452.0 ± 75.9	2,653 ± 82.1	+6.17	<0.0001

Signal intensities are given as mean ± standart deviation of four replicates. P values between four control replicates and four stress replicates for each probe were calculated using unpaired, 2-tailed student's t test

Osa: *Oryza sativa*, ppt: *Physcomitrella patens*, pta: *Pinus taeda*, ath: *Arabidopsis thaliana*, ptc: *Populus trichocarpa*, vvi: *Vitis vinifera*

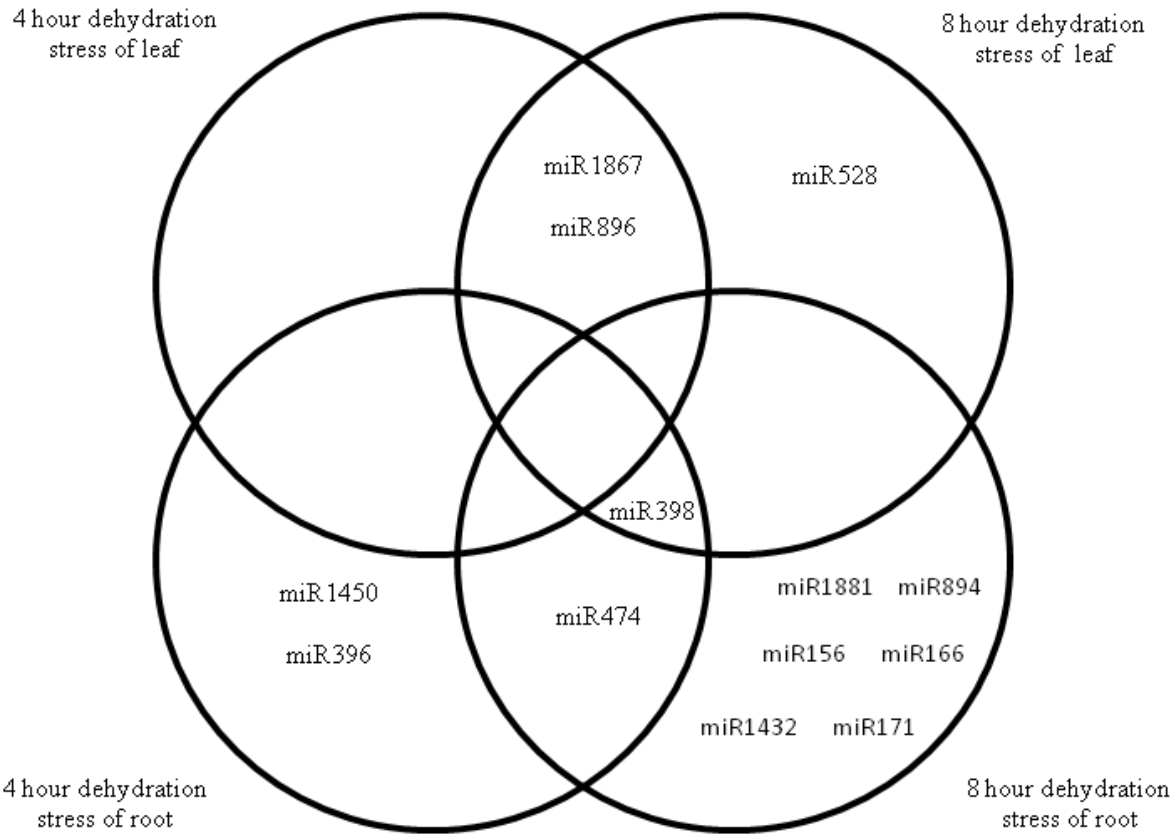

Figure 12 Venn diagram indicating common and unique differentially expressed wild emmer wheat microRNAs in two different tissues under two different drought treatments

In several cases multiple probes from the same family were detected as differentially expressed (miR398, miR474, miR156 and miR166). As miRNA family members usually differ from each other by only 2 or 3 bases, it is difficult to tell whether these multiple signals come from a single *T. dicoccoides* miRNA or several closely related ones. However, some indication is given by comparing their expression levels and cluster analysis (Figure 13). For example, all three miR474 probes were observed to be upregulated to a similar degree, suggesting that they are detecting the same *T. dicoccoides* miR474, or several miR474s that are regulated similarly. For miR-156 and -166 family members, although mean expression levels vary, they group together in the cluster analysis again suggesting a single corresponding *T. dicoccoides* miRNA or miRNA family. However, the different miR398 probes do not consistently cluster together, and show different levels of induction from each other (or no induction) depending on the drought condition. Therefore, it is probable that *T. dicoccoides* contains 2 or more miR398 family members that are induced under drought conditions but to different amounts depending on the tissue and drought conditions.

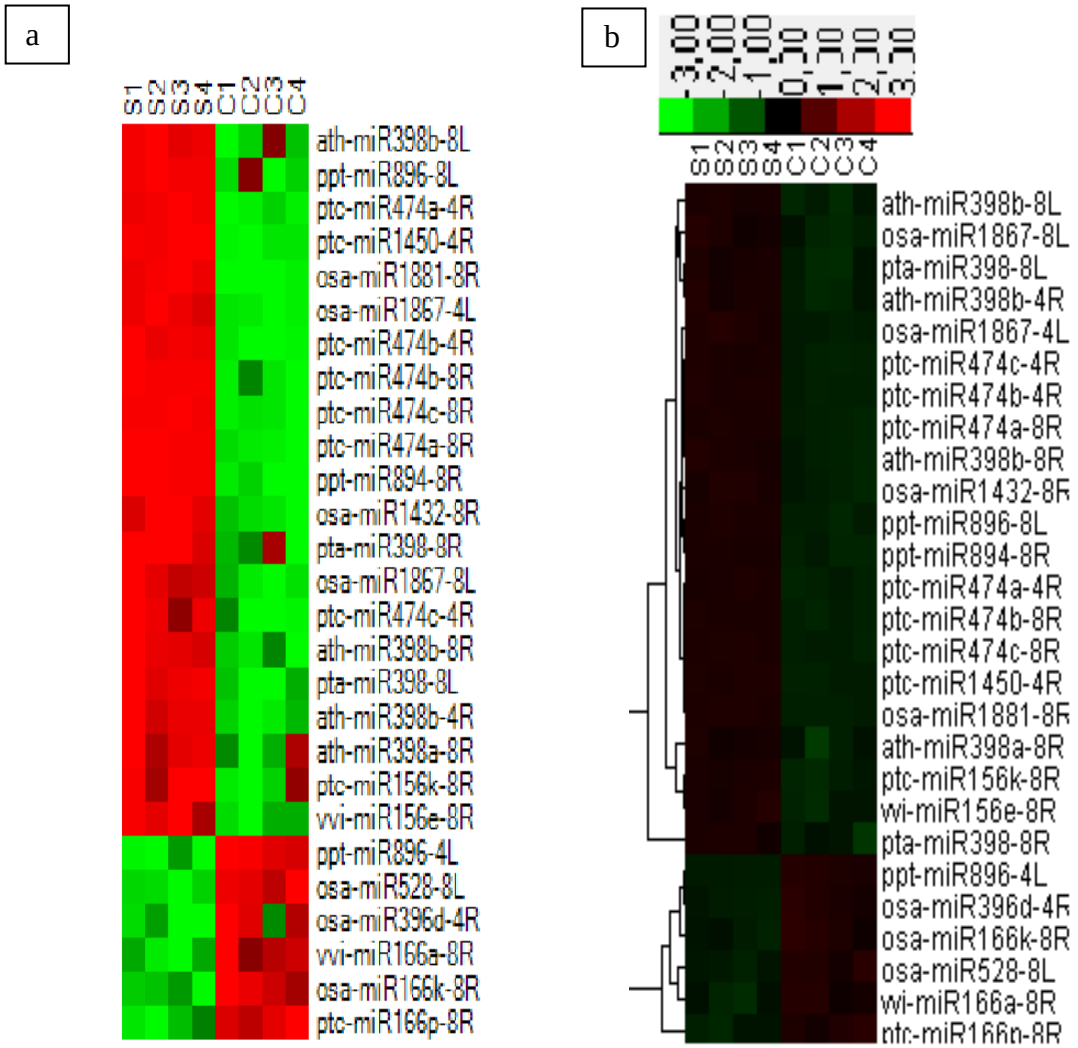

Figure 13 Heat map of microarray data showing differentially expressed wild emmer wheat microRNAs by tissue and drought treatment, clustered according to expression pattern. (a) clustering performed by the self-organizing map method using Euclidean distance in which green indicates low signal intensity and red high signal intensity (b) complete hierarchical clustering carried out using Euclidean distance in which color coding is according to the scale given 4L: 4 hour dehydration stress of leaf, 8L: 8 hour dehydration stress of leaf, 4R: 4 hour dehydration stress of root, 8R: 4 hour dehydration stress of root

4.2.3. Stem-loop Verification for Wild Emmer Wheat microRNAs

Although mature miRNA expression obtained by microarray studies is valuable, an ultimate verification for the presence of a miRNA in a species also necessiates pre-miRNA stem-loop evidence. This is crucial to differentiate miRNAs from other types of sRNAs since miRNAs are processed through a unique biogenesis pathway, in which hairpin shaped precursors are intermediates. Here, by searching sequences of all probes that gave a postive signal in the microarray, to wild emmer wheat ESTs, stem-loop forming secondary structures consistent with pre-miRNA characteristics were found for three miRNAs: miR1436, miR1128 and miR1132 (Figure 14). Three nucleotide mismatch flexibility was allowed to account for the occurences of miRNA-probe binding through a few mismatches and also taking into account differences in sequence between cultivars. The latter is important since microarray study was performed on a pool of TR39477 and TR38828, however in prediction of secondary structures, TR39477 and TTD-22 ESTs, the only currently available *T. dicoccoides* sequences, were used. Other miRNAs detected by the microarray await similar stem-loop evidence, which can be achived in the following years with an increase in *T. dicoccoides* genomic and transcriptomic sequence availibility.

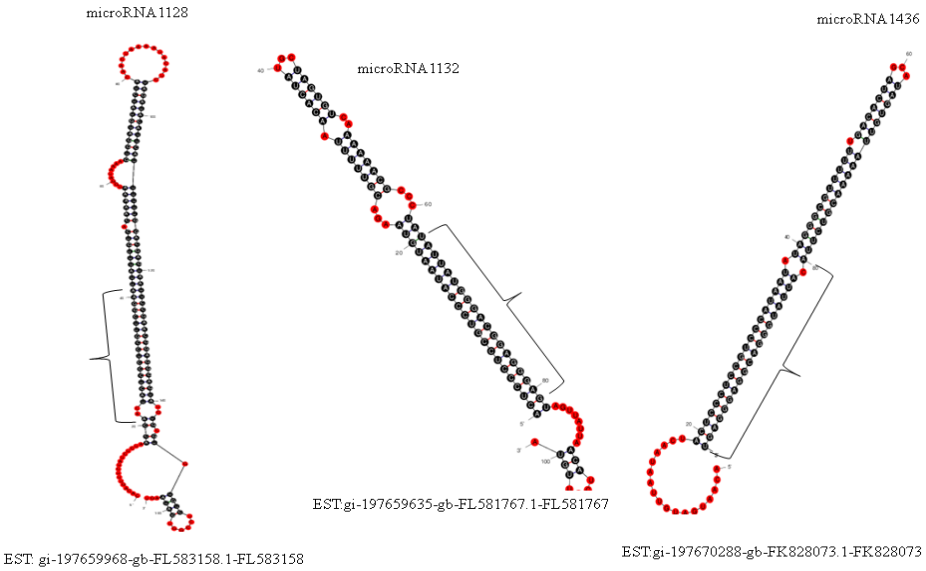

Figure 14 Predicted stem-loop structures of wild emmer wheat microRNAs identified by the microarray. Preliminary microRNA sequences were identified by BLAST to *T. dicoccoides* sequences NCBI (http://www.ncbi.nlm.nih.gov) (Ergen and Budak 2009) and their secondary structures were predicted by UNAFold.
Mature microRNA sequences are shown with brackets.

4.2.4. Putative Targets of Dehydration Responsive Wild Emmer Wheat microRNAs

Several of the differentially expressed miRNAs had experimentally confirmed targets in model plant species (Table 6). However, as the majority have not, we computationally predicted targets for all the differentially expressed miRNAs. Using wild emmer wheat sequences as the target dataset, only one predicted target was found for a differentially expressed miRNA: a putative heat-shock protein containing thioredoxin and DnaJ domains as a target of miR396. Therefore, the analysis was expanded to the set of available durum and bread wheat sequences. For miR156, miR166, miR396 and miR398, targets were predicted in *Triticeae* that matched those experimentally verified in model species (Table 6). For all the differentially expressed miRNAs except miR896, there was either a experimentally verifed target in model plant species, and/or a predicted target from *Triticum* ESTs or gene sequences.

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Table 6 Targets of drought responsive microRNAs identified by the microarray and their functions

Targets experimentally verified in other plant species and their functions			
microRNA	**Experimentally verified target**	**Reference**	**Target function**
miR398	copper superoxide dismutases; cytochrome C oxidase subunit V	Sunkar et al (2006); Jones-Rhoades & Bartel (2004)	copper homeostasis, response to oxidative stress, enzyme involved in respiration
miR474	kinesin, a pentatricopeptide repeat (PPR) family protein	Lu et al (2005)	motor functions, organelle biogenesis
miR166	HD-ZipIII transcription factors	Juarez et al (2004)	regulation of axillary meristem initiation and leaf development
miR156	squamosa-promoter binding protein (SBP)-like transcription factors	Wu & Poethig (2006)	development, especially flowering time
miR171	GRAS domain or SCARECROW-like proteins, a family of transcription factors	Llave et al (2002)	radial patterning in roots, light signaling, gibberellin signaling
miR396	growth regulating factor-like (GRL) transcription factors, ceramidase genes	Jones-Rhoades & Bartel (2004); Liu & Yu (2009)	development of leaves and cotydelons, lipid metabolism
Functional annotation of targets predicted using available *Triticum* sequences			
microRNA	**Source of sequence used in target prediction**	**Target description**	**Target function**
miR156	*Triticum aestivum*	squamosa binding protein-like	development, flowering time
miR166	*Triticum aestivum*	HD-ZipIII transcription factors	regulation of axillary meristem initiation, leaf development
miR396	*Triticum dicoccoides*	heat-shock protein containing thioredoxin and DnaJ domains	response to abiotic stress
miR396	*Triticum aestivum*	GRF-1 homologue	development of leaves and cotyledons
miR398	*Triticum durum*	Cu-Zn superoxide	copper homeostasis,

	Triticum aestivum	dismutases	response to oxidative stress
miR528	*Triticum aestivum*	similar to plantacyanin	guidance of pollen tubes into the style, reproduction
miR894	*Triticum aestivum*	similar to protein phosphatase PP2A-4	signalling pathways
miR1432	*Triticum aestivum*	phenylalanine tRNA synthetase-like	protein synthesis
miR1450	*Triticum durum*	Mn superoxide dismutases	response to oxidative stress
	Triticum aestivum		
miR1867	*Triticum durum*	putative protein, DUF1242 superfamily	-
	Triticum aestivum		

Sequences used for prediction consisted of *Triticum dicoccoides* expressed sequence tags (Ergen and Budak 2009), *Triticum durum* unique transcripts (PlantGDB, http://www.plantgdb.org/) and *Triticum aestivum* gene index release 9.0 (Lee et al. 2005).

4.3. Analysis of Bread Wheat 5D Chromosome microRNA Repertoire

4.3.1. Putative Bread Wheat 5D Chromosome microRNA Repertoire

Using the *in silico* homology-based method through implementation of in house perl scripts, 55 different bread wheat miRNAs were identified from chromosome 5D sequences. miRNA variety was observed to be slightly higher in the long arm (48 miRNAs; 13 specific to 5DL) compared to the short arm (42 miRNAs; 7 specific to 5DS). 35 miRNAs were detected to be common to both chromosome arms, revealing the presence of separate clusters in 5DS and 5DL encoding for the same miRNAs. Fourteen of a total of 55 predicted miRNAs were identified for the first time in bread wheat, of which 7 were found to be common to both chromosome arms, while 4 and 3 were specific to 5DS and 5DL, respectively (Table 7).

Table 7 List of identified putative bread wheat 5D chromosome microRNAs

List of microRNAs identified only in the short arm of bread wheat 5D chromosome			
miR3700	miR395	miR482	miR6224
miR834	miR845	miR950	
List of microRNAs identified only in the long arm of bread wheat 5D chromosome			
miR1123	miR160	miR169	miR2275
miR398	miR437	miR5085	miR5086
miR5161	miR5169	miR5281	miR6220
miR818			
List of microRNAs identified from both arms of bread wheat 5D chromosome			
miR1117	miR1118	miR1120	miR1121
miR1122	miR1125	miR1127	miR1128
miR1130	miR1131	miR1133	miR1135
miR1136	miR1137	miR1139	miR1436
miR1439	miR167	miR1847	miR2118
miR5021	miR5049	miR5067	miR5068
miR5070	miR5175	miR5180	miR5181
miR5203	miR5205	miR5387	miR5568
miR6191	miR6197	miR6219	

microRNAs identified for the first time in this research are underlined.

Each of the predicted miRNAs were observed to correspond to a variable number of putative pre-miRNA and mature miRNA sequences. Genomic representation in relation to different pre-miRNA coding genomic regions will be discussed in detail in Section 4.3.2. Regarding to mature miRNA sequences, a total of to 926 nonidentical were identified from 5D sequences, of which 654 and 428 were encoded from 5DL and 5DS, respectively. Mature miRNAs is size is crucial in terms of its function. Previous studies have shown that 22 nucleotide (nt) miRNAs are more likely to trigger small interfering RNA (siRNA) biogenesis from their target transcripts (Chen et al. 2010) and experimental analysis has revealed that 22 nt mature miRNA sequences were most effectively sorted and loaded onto AGO (Manavella, Koenig, and Weigel 2012). Here, we investigated the size distribution of 926 mature miRNAs encoded from 5D chromosome. Sizes of the majority of mature miRNAs predicted ranged between 20-24 nucleotides with an average of approximately 21,85 nucleotides (Figure 15).

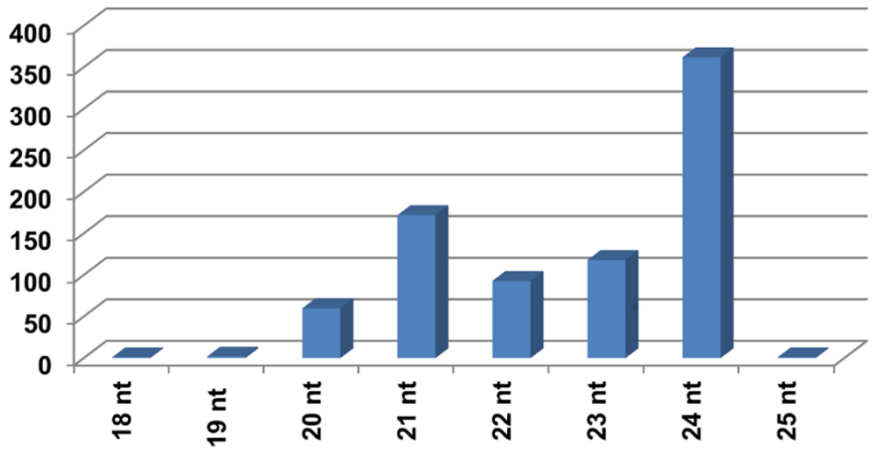

Figure 15 Mature microRNA length distribution of putative bread wheat 5D chromosome microRNAs. x-axis: mature microRNA length, y-axis: number of predicted bread wheat 5D chromosome mature microRNAs with the specified length nt: nucleotide

Predicted pre-miRNAs were observed to vary in sequence and stem-loop characteristics (Figure 16). For each chromosome arm, datasets containing one selected pre-miRNA sequence for each predicted miRNA were constructed to comparatively evaluate pre-miRNA statistics of 5DS and 5DL (Appendices M, N, O, P). By the cumulative assessment of these datasets containing 48 and 42 pre-miRNA sequences from 5DL and 5DS, respectively, pre-miRNA characteristics were found to correlate well with previous plant miRNA identification studies (Schwab et al. 2005; Unver and Budak 2009; Yin et al. 2008; Zhang et al. 2008; Zhang, Wang, and Pan 2007; Zhang, Wang, Wang, et al. 2007). Descriptive statistics in relation pre-miRNA sequence length and (G+C)% content; MFE values of pre-miRNA stem-loop secondary structures and MFEI indices are listed in Table 8 (refer to Section 4.1.1 for explanation of pre-miRNA characteristics). Although 5DL pre-miRNAs were detected to be slightly longer; due to their lower (G+C)% content and higher stability evident with more negative MFEs, they were observe to have MFEIs comparable those of 5DS pre-miRNAs, corresponding to an approximate average of 1,2. (Table 8).

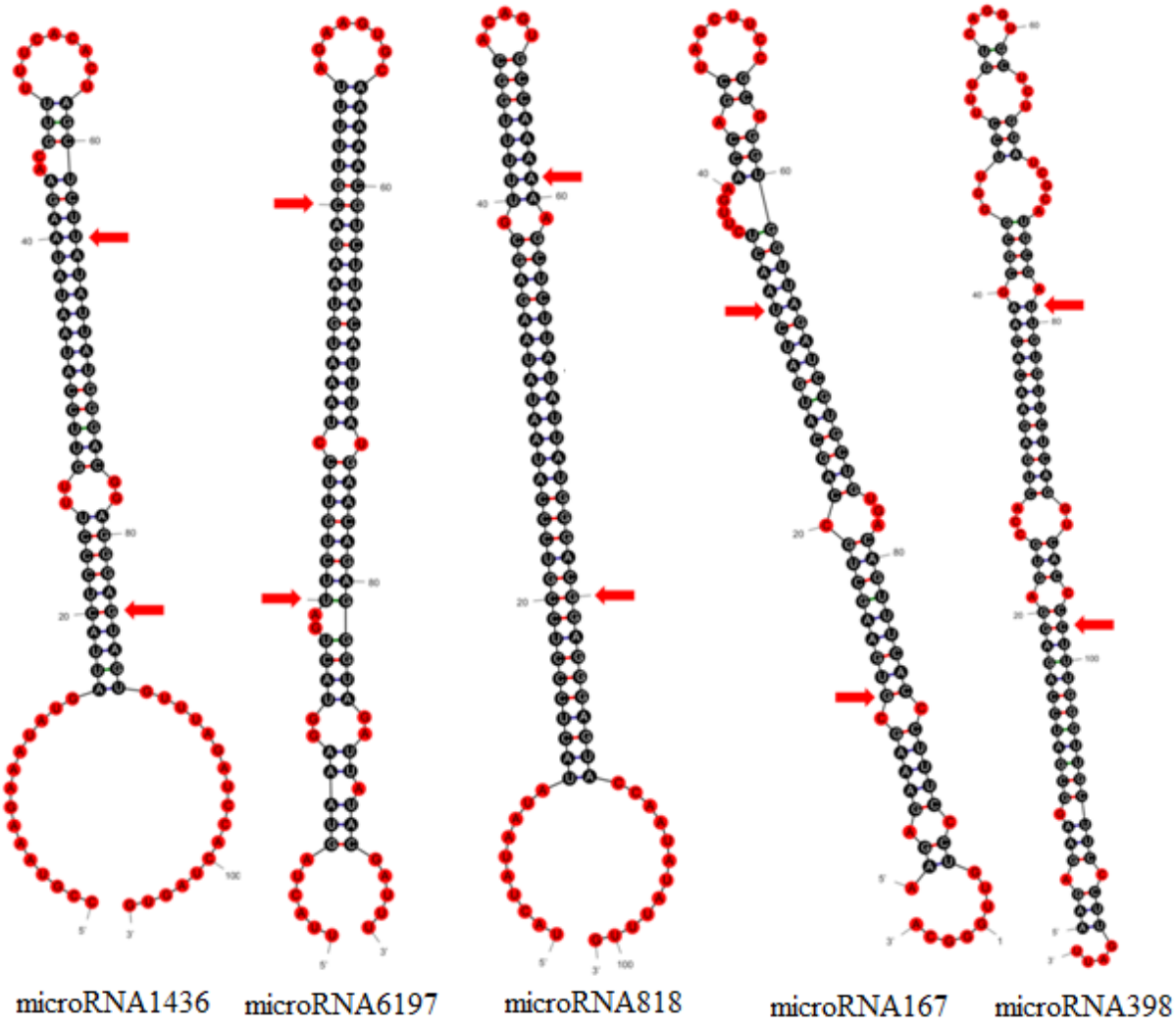

Figure 16 Predicted stem-loop structures of selected putative bread wheat 5D chromosome microRNAs. Mature microRNA start and end positions are designated with arrows. Structures were predicted using UNAFold.

Table 8 Statistics for selected putative preliminary microRNAs of short and long arms of bread wheat 5D chromosome

For the combination of preliminary microRNA datasets of short and long chromosome arms of 5D chromosome				
	Sequence length	**ΔG (kcal/mol)**	**G+C (%)**	**MFEI**
Average	130.02	-61.83	40.46	1.2
Minimum	74	-161.5	26.07	0.68
Maximum	291	-23.4	67.01	2.08
Median	122	-59.7	38.02	1.18
Standard deviation	39.39	23.44	8.61	0.27
For the preliminary microRNA dataset of the long arm of 5D chromosome				
	Sequence length	**ΔG (kcal/mol)**	**G+C (%)**	**MFEI**
Average	136.88	-63.35	39.6	1.2
Minimum	90	-161.5	26.07	0.72
Maximum	291	-29.2	59.41	2
Median	130	-61.3	37.69	1.16
Standard deviation	46.61	28.21	8.83	0.3
For the preliminary microRNA dataset of the short arm of 5D chromosome				
	Sequence length	**ΔG (kcal/mol)**	**G+C (%)**	**MFEI**
Average	122.19	-60.08	41.5	1.2
Minimum	74	-134.4	27.84	0.68
Maximum	273	-23.4	67.01	2.08
Median	117	-58.05	39.2	1.19
Standard deviation	34.32	21.42	8.73	0.27

ΔG: minimum folding free energy, G+C: guanine+cytosine percent of sequence, MFEI: minimum folding free energy index

4.3.2. Genomic Representation of Putative Bread Wheat 5D Chromosome microRNA Repertoire

A total of 3143 genomic loci coding for putative 55 miRNAs were predicted to be present on 5D chromosome. Long arm was observed to contain a higher genomic representation of miRNAs compared to the short (1952 for 5DL; 1191 for 5DS), as might be expected from its larger size. The differences in the amount of representation of individual miRNAs on 5D were found to be highly variable. For instance, representation of miR5049 coding loci on 5D was observed to be as high as 326: 120 and 206 from the short and long arms, respectively. Other 5D miRNAs with the highest apparent representation (over 200 copies) were miR1117, miR1120, miR1122 and miR1436, with variable, but high contributions to their representation from two chromosome arms. On the other hand, some miRNAs had low genomic representation. In fact, eleven miRNAs were only detected at a single locus throughout the whole chromosome: 7 on 5DS and 4 on 5DL. Two genomic coding loci was identified for miR5070, one on the long and one on the short arms. Four others miRNAs were also represented by only one genomic locus on 5DS, but that had higher folds on 5DL. Although this analysis does not render the determination of absolute copy number of each miRNA with certainity since some genomic miRNAs may be covered by more than one sequence read, while others may not be covered at all. However, the representation of each miRNA within the dataset provides a useful estimate of its prevalence on the chromosome. It is also noteworthy that 11 of the 14 newly identified miRNAs were observed to have representations lower than 20 copies. Up until now, they may have been overlooked in expression based studies due to their low representation (Table 9).

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Table 9 Representation of putative microRNA coding regions on bread wheat short and long chromosome arms separately and cumulatively

microRNA	5DS	5DL	5D	microRNA	5DS	5DL	5D
miR1117	117	176	293	**miR482**	1	0	1
miR1118	1	22	23	**miR5021**	12	7	19
miR1120	101	131	232	**miR5049**	120	206	326
miR1121	8	3	11	**miR5067**	14	24	38
miR1122	80	160	240	**miR5068**	1	9	10
miR1123	0	3	3	**miR5070**	1	1	2
miR1125	1	24	25	**miR5085**	0	1	1
miR1127	38	45	83	**miR5086**	0	1	1
miR1128	53	65	118	**miR5161**	0	6	6
miR1130	56	90	146	**miR5169**	0	4	4
miR1131	47	105	152	**miR5175**	18	11	29
miR1133	7	25	32	**miR5180**	7	14	21
miR1135	32	120	152	**miR5181**	18	27	45
miR1136	31	113	144	**miR5203**	10	3	13
miR1137	64	59	123	**miR5205**	25	60	85
miR1139	106	56	162	**miR5281**	0	5	5
miR1436	119	146	265	**miR5387**	15	80	95
miR1439	34	42	76	**miR5568**	11	7	18
miR160	0	4	4	**miR6191**	3	10	13
miR167	4	2	6	**miR6197**	12	25	37
miR169	0	9	9	**miR6219**	1	9	10
miR1847	12	11	23	**miR6220**	0	3	3
miR2118	5	5	10	**miR6224**	1	0	1
miR2275	0	1	1	**miR818**	0	17	17
miR3700	1	0	1	**miR834**	1	0	1
miR395	1	0	1	**miR845**	1	0	1
miR398	0	1	1	**miR950**	1	0	1
miR437	0	4	4				

microRNAs identified for the first time in this research are underlined. 5DS: short arm of bread wheat 5D chromosome, 5DL: long arm of bread wheat 5D chromosome

4.3.3. Repeat Content of Putative Bread Wheat 5D Chromosome microRNA Repertoire

The high representation of some of the putative miRNAs detected on chromosome 5D suggests that some or all of their genomic loci could be repetitive sequences. *Triticeae* genomes are known to be highly repetitive (85-90%) (Lucas et al. 2012, 2013) and repeat related activities were observed to result in new gene and pseudogene formation, contributing to genome evolution (Wicker et al. 2011). Besides, recent evidence suggests that miRNA gene evolution is also driven by the activities of transposons (Li et al. 2011). Therefore, all putative pre-miRNA hairpin sequences detected above were searched against a database of known repetitive elements. Interestingly, the long arm stem loop sequences were masked with repeats slightly less (83.84%) compared to those on the short arm (84.38%). The distribution of repeat elements also showed variation between 5DL and 5DS. Such differences in composition and distribution of transposable elements between different chromosomes, and even distinct regions of the same chromosome in wheat species have been reported previously (Li et al. 2004; Paux et al. 2006; Sabot et al. 2005). In here, pre-miRNA hairpins were found to be rich especially for DNA transposons, encompassing 81.54% of 5DL and 81.98% of 5DS stem-loop coding sequences. However, for retrotransposons these values were only 0.03% and 0.16% corresponding to 5DL and 5DS, respectively, indicating low retrotransposon representation in stem-loop coding loci. The most predominant Class II transposon families identified in the stem-loops were TcMariner (TcMar) and Enhancer/Suppressor-mutator (En/Spm) for both chromosome arms. Tourist/Harbinger/Stowaway subfamily was also detected in the stem-loops of both arms with a much higher represention in 5DS pre-miRNAs. Repeats of this family were specifically found to be located in miR1139 and miR5049 encoding stem-loops. Additionally, Mutator (MuDR) elements were found to be present in the stem-loops of 5DL, but not in those located on 5DS. Regarding to Class I transposon families, 5DL stem-loops were observed to contain long interspersed nuclear elements (LINE), as well as long terminal repeat (LTR) retrotransposons of the Gypsy subfamily. On the contrary, 5DS stem-loops were not detected to harbour LINEs, but found to harbour LTR retrotransposon of both Copia and Gypsy subfamilies. Hairpins were also identified to encompass other non-transposon repeat elements including simple repeats (Figure 17).

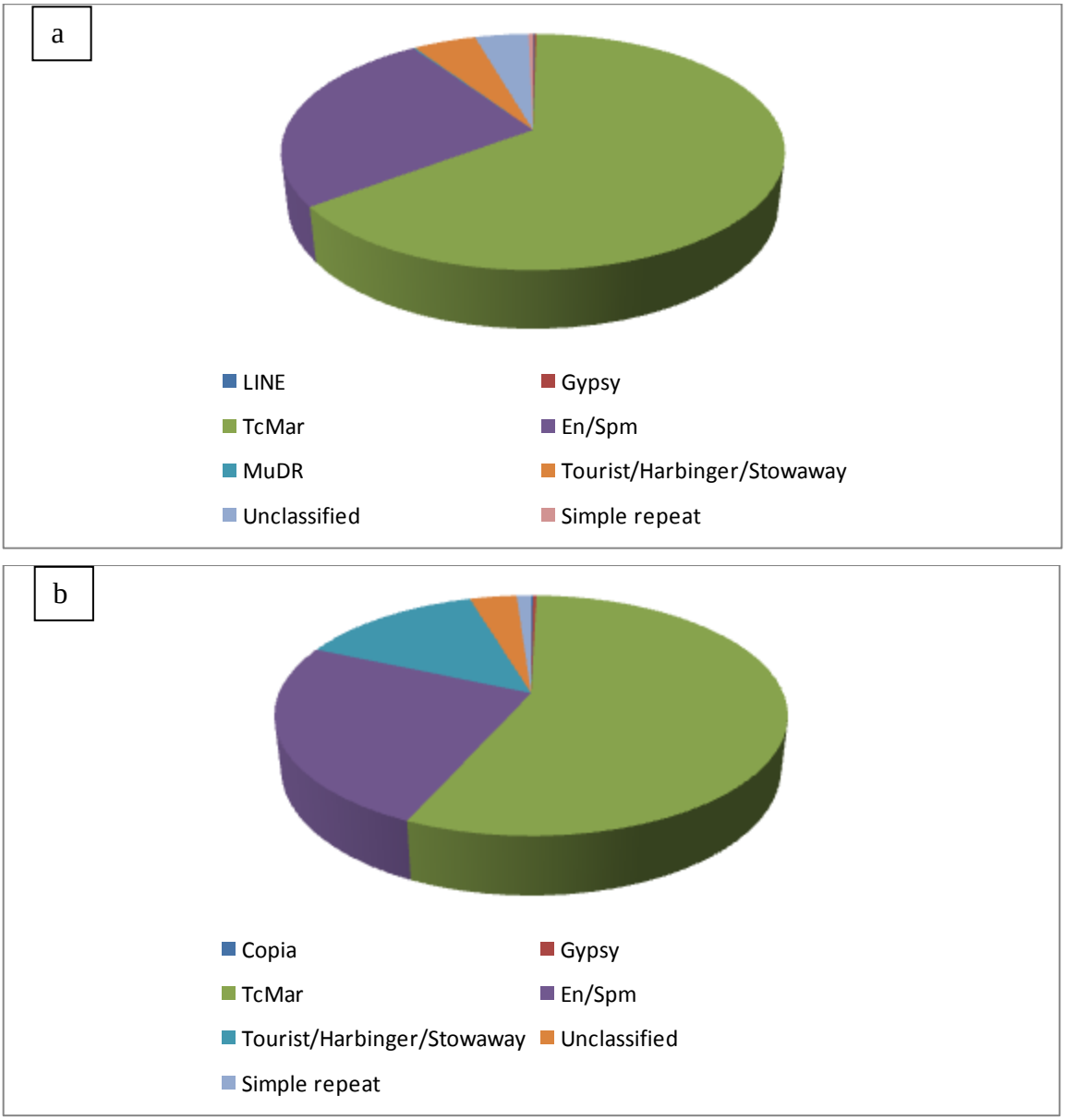


Figure 17 Distribution of repeats of distinct subfamilies in putative bread wheat 5D chromosome preliminary microRNA repertoire (a) for the long chromosome arm microRNAs (b) for the short chromosome arm microRNAs
LINE: long interspersed nuclear element, TcMar: TcMariner, En/Spm: Enhancer/Suppressor-mutator, MuDR: Mutator

Overall, thirty-six out of 55 predicted miRNAs were detected to contain repeats of the DNA transposon family. Since retrotransposons are known to constitute the major portion of repetitive elements in plant genomes, the accumulation of Class II elements in miRNA coding regions is noteworthy and supports the view that Class II elements contribute to miRNA evolution (Li et al. 2011). Pre-miRNAs and their corresponding DNA transposon content is listed in Table 10.

Table 10 Putative bread wheat 5D chromosome microRNAs and DNA transposon contents of their preliminary microRNA sequences

microRNA	Repeat families	microRNA	Repeat families
miR1117	En/Spm	**miR437**	TcMar, En/Spm
miR1118	TcMar, En/Spm	**miR5021**	En/Spm
miR1120	TcMar, En/Spm	**miR5049**	TcMar, En/Spm, Stowaway
miR1121	TcMar	**miR5067**	TcMar
miR1122	TcMar	**miR5161**	TcMar
miR1123	MuDR	**miR5169**	TcMar
miR1125	TcMar	**miR5175**	TcMar
miR1127	TcMar, En/Spm	**miR5180**	TcMar
miR1128	TcMar, En/Spm	**miR5203**	TcMar
miR1131	En/Spm	**miR5205**	TcMar, En/Spm
miR1133	TcMar	**miR5281**	TcMar, En/Spm
miR1135	TcMar	**miR5387**	En/Spm
miR1136	TcMar	**miR5568**	TcMar
miR1137	TcMar	**miR6191**	TcMar
miR1139	Harbinger	**miR6219**	En/Spm
miR1436	TcMar, En/Spm	**miR6224**	TcMar, En/Spm
miR1439	TcMar, En/Spm	**miR818**	TcMar
miR1847	En/Spm	**miR834**	En/Spm

TcMar: TcMariner, En/Spm: Enhancer/Suppressor-mutator, MuDR: Mutator

4.3.4. Putative Targets of Predicted Bread Wheat 5D Chromosome microRNAs

All of the putative wheat miRNAs on chromosome 5D were found to have predicted or experimentally confirmed targets, involved in biological or metabolic processes and in stress responses. For all putative 55 miRNAs, *in silico* targets were identified (Appendices R and S), yet only 3 had homologs in other species with confirmed targets (Table 11). Putative wheat miRNA target genes varied in sequence and function, and most of them were classified as transcription factors, functional proteins in plant metabolism, and protein subunits (Figure 18). Predicted targets of

newly identified miRNAs were involved in a broad range of biological and molecular functions: including hydrolase activity (miR3700: TC412324), nucleic acid binding transcription factor activity (miR5205: TC413453), transferase activity (miR5568: TC446402, TC395950), oxidoreductase activity (miR482: CO348589), metal ion binding activity (miR6197: AL821953) and response to stresses (miR5387: BE637541) such as drought. The majority of miRNAs were observed to have more than one potential regulatory target, while one target could be regulated by more than one miRNA. This observation supports the idea that miRNA studies should focus on a regulatory network in which more than one miRNA with different targets are involved.

Table 11 miRBase deposited experimentally confirmed targets for homologs of bread wheat 5D chromosome microRNAs

microRNA	**Species in which the target was identified**	**Target**
miR167	ath/osa	auxin response factors
miR395	ath/osa	ATP sulphurylase
miR160	ath/osa	auxin response factors

ath: *Arabidopsis thaliana*; osa: *Oryza sativa*
miRBase (version 19, August 2012, http://www.mirbase.org/) (Kozomara and Griffiths-Jones 2011)

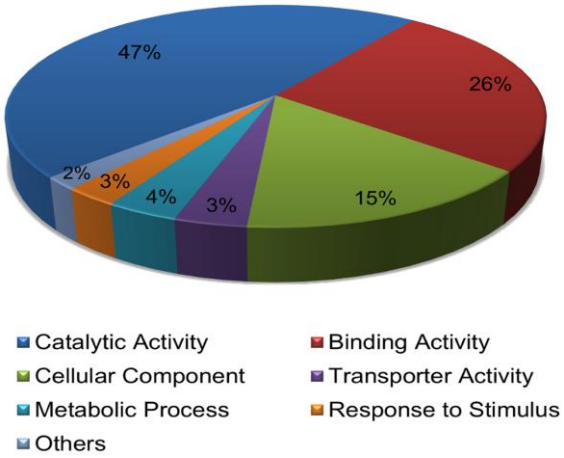


Figure 18 Distribution of predicted target functions of putative bread wheat 5D chromosome microRNAs. Piechart was constructed based on predicted targets of 14 newly identified microRNAs. DFCI Gene Index Release 12 (http://plantgrn.-noble.org/psRNATarget/) was used as the target sequence dataset. QuickGO provided by UniProt-GOA project at EBI (http://www.ebi.ac.uk/QuickGO/) was used for annotation.

4.3.5. Computational Evidence for Expression of Predicted Bread Wheat 5D Chromosome pre-microRNAs

Unlike other sRNAs, miRNAs are generated from pri-miRNA transcripts, which are capped and polyadenylated in the same manner as protein-coding mRNAs (Lee et al. 2004). Therefore, pri-miRNA sequences may be found in EST databases, albeit rarely (Dryanova et al. 2008). Here, in order to provide expression evidence for putative miRNAs; non-protein coding, candidate pre-miRNA coding ESTs that show high similarity to our dataset of predicted pre-miRNA sequences were determined. Through searches 6 miRNAs matched an EST with no significant similarity to known proteins, suggesting that these putative pre-miRNA sequences are transcribed (Table 12). The remaining putative pre-miRNAs may also be transcribed, but absent from the currently available EST databases.

Table 12 List of expressed sequence tags that have high homology to putative bread wheat 5D chromosome microRNAs

microRNA	Expressed sequence tag
miR1122	CJ632148.1
miR1439	CJ510559.1
miR1436	AL816538.1
miR167	CJ846906.1, CJ833771.1
miR5205	CJ631979.1, CJ523432.1
miR1136	CJ665546.1, CD876589.1, BE591362.1

Hits with a query coverage or maximum identity lower than 99% and 98%, respectively or with similarity to a protein coding sequence at an e-value equal to or lower than 1E-03 were eliminated. Expressed seqeunce tag: Genbank (NCBI (January 2013) accession ID of the expressed sequence tag

4.3.6. Localization and Quantification of pre-microRNA Coding Regions on Bread Wheat 5D Chromosome

In order to verify 5D chromosome localization, five of the predicted pre-miRNA coding regions were amplified from flow sorted 5D chromosome arms by PCR. screening. Our experimental results supported our in *silico* predictions: 5DS was verified to harbour regions coding for pre-miR2118 and pre-miR5070, and 5DL was confirmed to contain both of the above plus pre-miR6220, pre-miR5085 and pre-miR169 coding regions. Furthermore,in order to confirm that these pre-miRNAs are specifically located on chromosome 5D, we also screened gDNA from Chinese Spring and nullitetrasomic lines. pre-miR169, pre-miR5085 and pre-miR2118 coding regions were found to be 5D chromosome specific. miR2118 was shown to be located on both arms of the 5D chromosome (5D specific), while miR5085 and miR169 were found to be specific to the long arm (Figure 19).

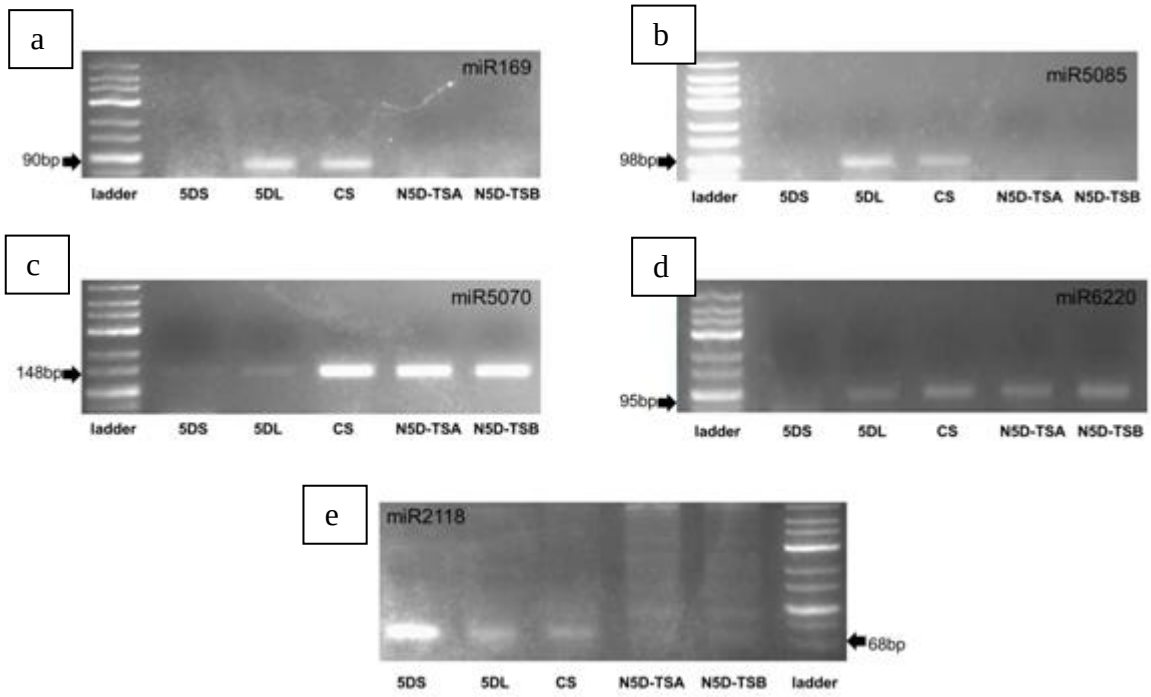


Figure 19 Screening for the specific presence of preliminary microRNA coding regions on long and short arms of bread wheat 5D chromosome. (a) microRNA169 (b) microRNA5085 (c) microRNA5070 (d) microRNA6220 (e) microRNA2118
ladder: DNA ladder, 5DS: flow sorted bread wheat 5D chromosome short arm, 5DL: flow sorted bread wheat 5D chromosome long arm, CS: bread wheat cultivar Chinese Spring, N5D-T5A, N5D-T5B: bread wheat nullitetrasomic lines that lack 5D chromosome
For screening, PCR was performed and products were visualized through agarose gel electrophoresis.

Furthermore, to map the chromosomal positions of 5DL specific miRNAs, gDNA of group-5 deletion lines were also screened. Coding regions of both 5DL specific pre-miRNAs (pre-miR5085, pre-miR169) were found to be located between the breakpoint of 5DL-7 (FL : 0.29) and the centromere (Figure 20).

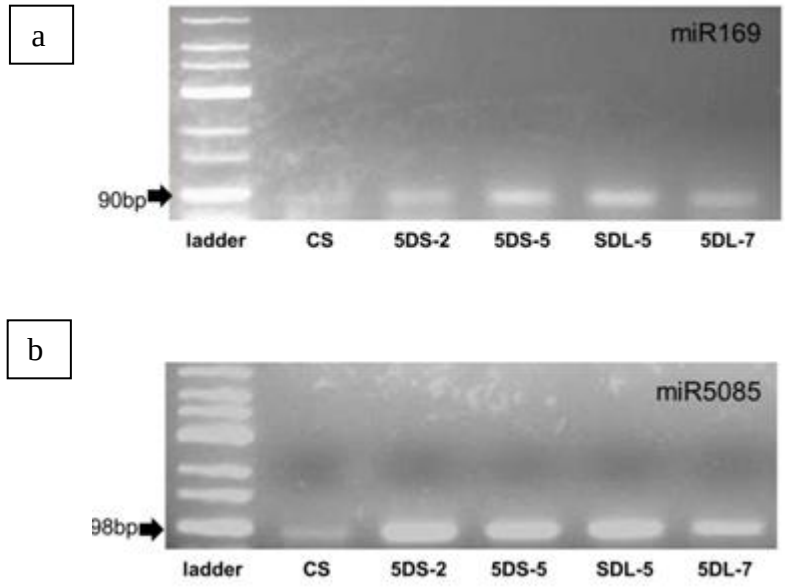


Figure 20 Screening for the location of preliminary microRNA coding regions on short and long arms of bread wheat 5D chromosome. (a) microRNA169 (b) microRNA5085 ladder: DNA ladder, CS: bread wheat cultivar Chinese Spring, 5DS-2, 5DS-5, 5DL-5, 5DL7: lines of bread wheat group-5 deletion series with different fraction length values For screening, PCR was performed and products were visualized through agarose gel electrophoresis

Quantification with real-time PCR using Chinese Spring gDNA suggested that coding regions of the selected pre-miRNAs had variable copy number: pre-miR169, pre-miR5085 and pre-miR5070 were shown to have approximately 86, 2.2 and 1.5 fold more copies than pre-miR6220. Gene copy number of pre-miR6220 was also separately evaluated with qRT-PCR in nullitetrasomic lines in comparison to Chinese Spring, to determine its gene copy number restricted to the 5D chromosome. Approximately 9% of pre-miR6220 coding sequence copies from the whole wheat genome were observed to be located on chromosome 5D (Figure 21).

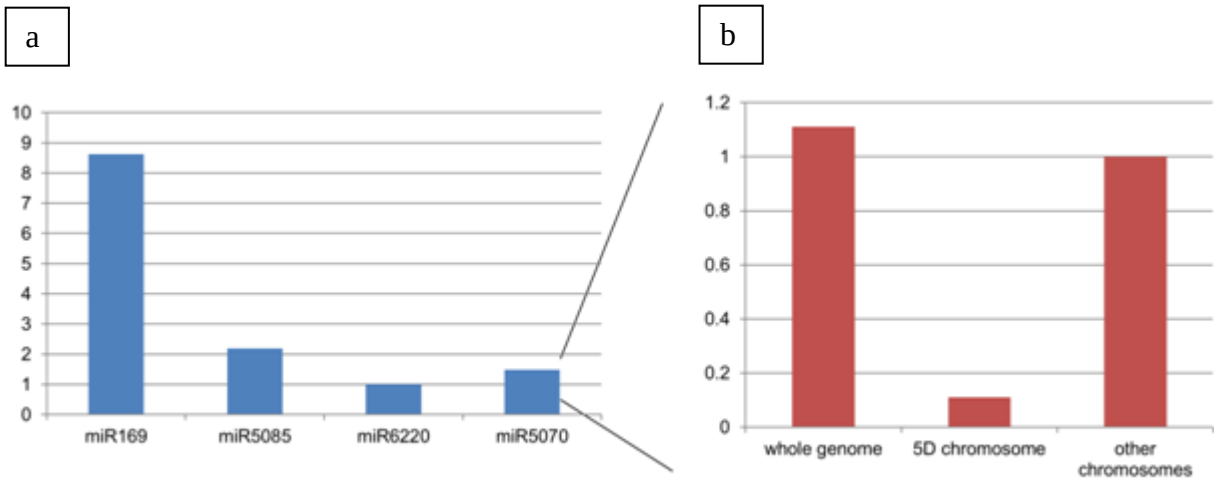


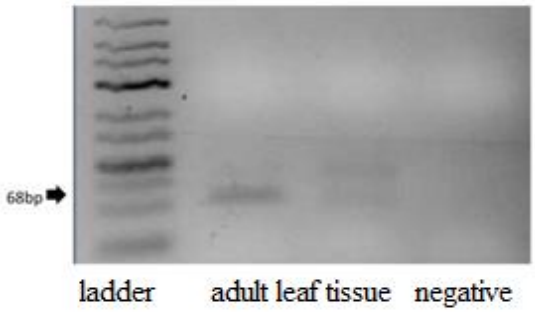
Figure 21 Quantification of gene copy number of preliminary microRNA coding regions. (a) comparative levels of microRNA169, microRNA5085, microRNA6220 and microRNA5070 coding regions in bread wheat cultivar Chinese Spring (b) comparative levels of microRNA5070 coding regions on 5D chromosome and other bread wheat chromosomes with its quantification in nullitetrasomic line N5DT5A and bread wheat cultivar Chinese Spring
Quantification is based on quantitative real time PCR results and designated with bar graphs.

4.3.7. Expression of pre-micro2118 from Bread Wheat 5D Chromosome

In order to show expression of selected pre-miRNAs (pre-miR2118, pre-miR169, pre-miR5085, pre-miR6220, premiR5070), RT-PCR and qRT-PCR was performed using Chinese Spring cDNA. Expression of pre-miR2118 in adult leaves of wheat, grown under standard greenhouse conditions was shown. Expression was not unequivocally confirmed for the other 5D specific pre-miRNAs, but as individual miRNA expression is frequently tissue/developmental stage/environmental condition specific, their expression may be detectable under specific conditions that were not tested here, most probably stress
conditions (Figure 22).

a

b



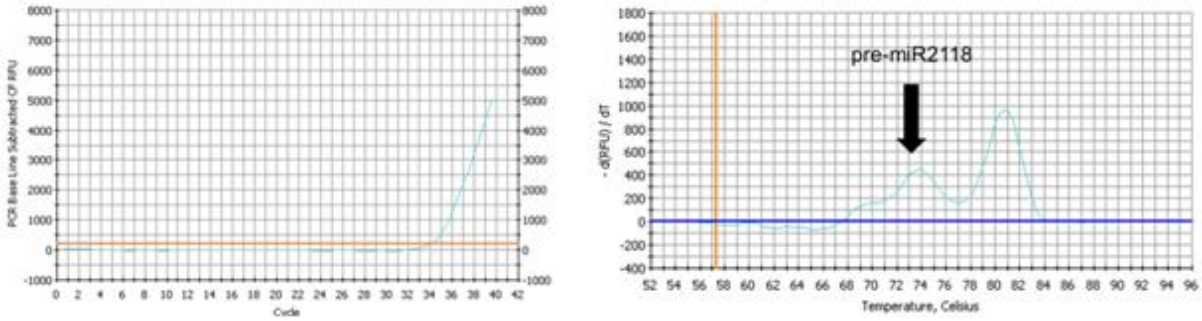


Figure 22 Evidence for preliminary microR2118 expression in bread wheat adult leaves.
(a) agarose gel electrophoresis of samples amplified by endpoint PCR (b) quantitative real time PCR amplification and melting curves
Experiments were carried out in bread wheat cultivar Chinese Spring.
ladder: RNA ladder

4.4. Introgression of Drought-related Quantitative Trait Loci to Elite Cultivars

4.4.1. Establishment of F1 Plants Carrying Drought-related Quantitative Trait Loci

Here, we introgressed drought related genomic regions from two South Australian cultivars into European elite germplasm. For this purpose, we performed all different possible combinations of intercultivar crosses between Australian cultivars (Kukri and RAC875) and public elite lines (Tosunbey and Bolal). Rate of plant development was observed to vary between varieties and in one trial approximate heading times were recorded as 42 days for Kukri, 47 days for Tosunbey, 51 days for Bolal and 53 days for RAC875.

Several seeds were obtained from cross-pollinated females after intercultivar crosses. Viable F1 plants were screened for successful introgression of drought related key regions on chromosome 3B from Kukri or RAC875, depending on the cross performed, using a linked SSR marker, XBARC77**.** We confirmed the successful introgression of drought related key regions in seven F1 genotypes from crosses between Bolal female X Kukri male (3 plants); RAC875 female X Tosunbey male (3 plants) and Tosunbey male X Kukri female (1 plant) (Figure 23).

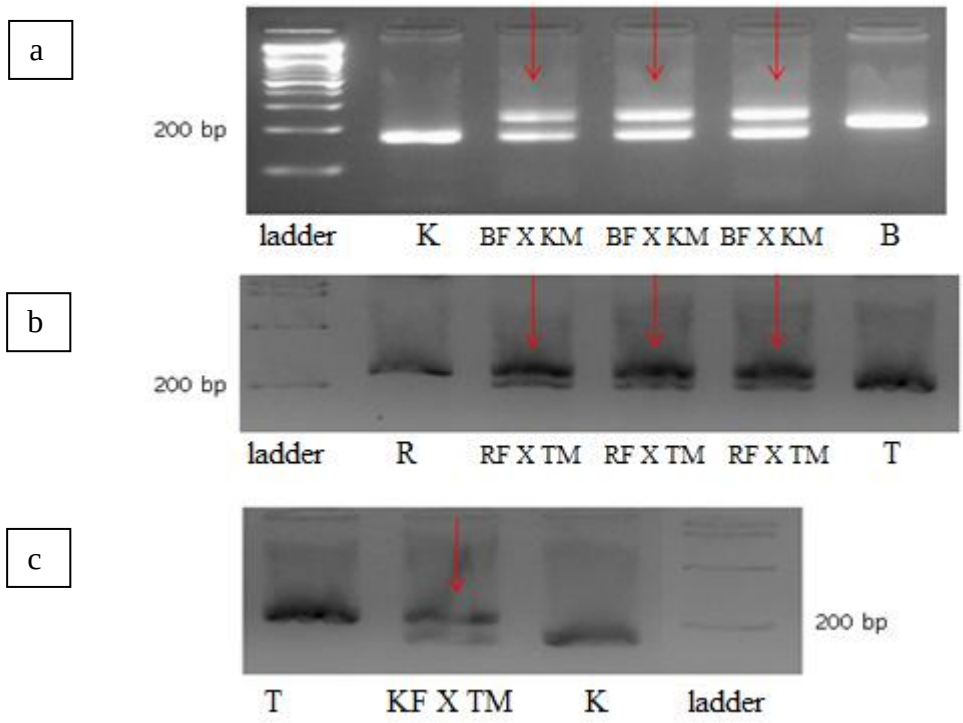


Figure 23 Amplification of XBARC77 simple sequence repeat marker in filial 1 plants. (a) three Bolal female X Kukri male plants (b) three RAC875 female X Tosunbey male plants (c) Tosunbey male X Kukri female plant
ladder: DNA ladder, K: Kukri, B: Bolal, R: RAC875, T: Tosunbey, F: female, M: male
Screening was performed with PCR and products were visualized by agarose gel electrophoresis.

4.4.2. Establishment of BC3F2 Plant Carrying Drought-related Quantitative Trait Locus

In order to limit the loss of good yield characteristics from the elite cultivar, obtained plants carrying the drought related key regions (refer to Section 4.4.1) were backcrossed against their parental backgrounds until BC3F2. BC3F2 plants carrying the drought related key region on chromosome 3B were obtained from backcrossing of obtained Bolal female X Kukri male F1 plants (Figure 24). These plants are promising for high performance in drought prone environments and are available for further experimental field testing.

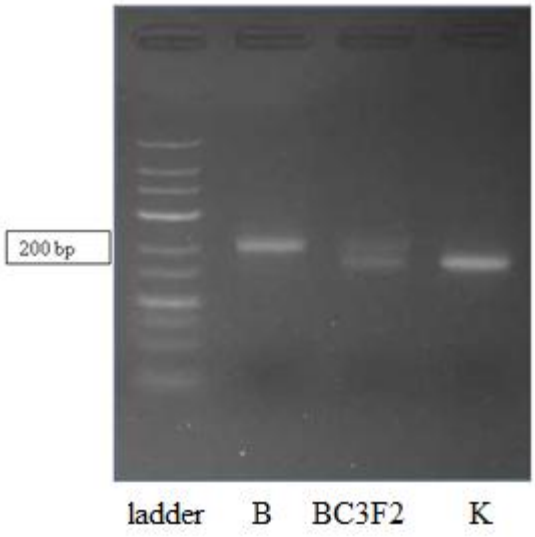


Figure 24 – Amplification of XBARC77 simple sequence repeat marker in backcross 3 filial 2 plants succeded from Bolal female X Kukri male filial 1 plants
ladder: DNA ladder, B: Bolal, BC3F2: backcross 3 filial 2 plants succeded from Bolal female X Kukri male filial 1 plants, K: Kukri
Screening was performed with PCR and products were visualized by agarose gel electrophoresis.

5.

DISCUSSION

Wheat and its related species are of great economic value, yet their domestication followed by selective breeding for higher yield has resulted in the loss of valuable alleles for drought tolerance in modern wheat. Drought is arguably the single abiotic factor threating wheat yields and its occurence is increasingly emphasized globally due to environmental warming. In order to stabilize wheat yields, an important consideration is the improvement of modern cultivars to perform better in water limited environments. To this end, elucidation of molecular mechanism of drought response and the effective transfer of acquired knowledge to breeding programmes are crucial.

miRNAs are central to post-transcriptional regulation of gene expression and have been shown to be involved in responses to a variety of stress factors including drought. Suprisingly, miRNA regulatory networks in *Triticeae* in the context of drought has been majorly overlooked. Here, we report drought related miRNAs and their respective targets in two different *Triticeae* species: barley and wild emmer wheat. Of these, barley, widely adapted to diverse environmental conditions, is more tolerant to drought compared to its close relative wheat. Wild emmer wheat holds particularly great value with its superior stress tolerance harboring valuable novel mechanisms lost during transition to modern wheat.

In this research, drought related miRNA regulatory mechanisms were observed to be highly tissue specific and effected by the severity of stress treatments. Besides, comparative analysis of drought induced fluctuations in miRNA repertoires across barley and wild emmer wheat pointed to both conserved and unique patterns of drought tolerance. The expressions of all four drought responsive miRNAs (miR408, miR171, miR156 and miR166) identified in barley, were also significantly altered in wild emmer wheat in response to drought. Although, the modes and/or levels of drought induced changes of some of these miRNAs were also detected to be paralel and/or comparable across species, this research also highlighted several differential tissue- and stress treatment- specific regulatory mechanisms exerted by homolog miRNAs in two different species. Putative drought related miRNAs identified in this research are available for further functional studies to the end of elaborating common and novel drought regulatory mechanisms in different *Triticaea* species and use of this knowledge for the development of cultivars with better performance under water deficiency.

In addition to identifying drought related miRNAs, an equally important aspect is the dissection of complete miRNA repertoires of plants and their careful evaluation in the genomic and/or subgenomic contexts. In recent years, bread wheat miRNAs expressed in different tissues, at distinct developmental stages and under certain environmental conditions were reported. However, results retrieved from these sRNA library- or microarray- based studies do not represent the whole miRNA repertoire, since miRNA expression is highly tissue specific and extensively regulated through development and by stress conditions. Hence, in order to assess the overall bread wheat 5D chromosome miRNA content, we systematically predicted the whole catalogue of miRNA coding regions on this chromosome with *in silico* analysis of high-throughput genomic sequence data. Fifty-five miRNAs were predicted to have coding regions on 5D chromosome. Genomic representations of different putative 5D chromosome miRNAs were observed to be highly variable: possibly an important factor in the degree of corresponding mature miRNA expression and its target regulation. Coding regions of miRNAs with high genomic representations were detected to be rich for DNA transposons, supporting the view that transposon activities contribute to MIR evolution. Approach adopted in this research: systematic *in silico* miRNA prediction from high-throughput genomic sequence data, followed by localization and quantification of miRNA coding regions on 5D chromosome through screening of nullitetrasomic and

chromosome deletion lines, provides a basis to the end of elaborating the complete bread wheat repertoire.

Although elucidation of drought related molecular pathway components is important, the ultimate goal of related research is to transfer such knowledge effectively to breeding programs. Hence, here we also introgressed drought related key genomic regions on chromosome 3B identified in South Australian bread wheat cultivars into European elite bread wheat lines. We confirmed the successful transfer of the region from Kukri to Bolal and from RAC875 to Tosunbey in newly obtained bread wheat genotypes using a linked SSR marker. Furthermore, in order to limit the loss of good yield characteristics from the elite cultivar, backcrossing was performed until BC3F2 for Bolal X Kukri genotype. These newly developed genotypes are promising for high performance under drought prone environments. Future directions include extensive characterization of the obtained plants for their yield components and water use efficiency under water limited conditions in the greenhouse and further multienvironment field testing.

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6.

CONCLUSION

Comparative miRNA repertoires reported here hold valuable information regarding to conserved or unique patterns of molecular response to stress. This information will be convenient for further functional studies expanding our understanding of gene regulation across *Triticaea* and the role of miRNAs in drought tolerance. Besides, new bread wheat genotypes obtained by introgression of drought related QTLs, identified in drought related Australian cultivars, to elite germplasm are highly promising in terms of their performance and await to be tested in terms of physiological parameters and in multiple fields.

APPENDIX A

Chemicals and Enzymes

6X DNA Loading dye	Thermo Scientific	R0611
Absolute ethanol	Riedel de Haen	32221
Agarose	PRONA	8016
Ampicillin	Sigma	A9393
Boric acid	Sigma	B6768
Calcium chloride ($CaCl_2$)	Sigma	B6768
Calcium nitrate ($Ca(NO_3)_2$)	Sigma-Aldrich	237124
Chloroform	Merck	102.445
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	40718
Dioxyribonuclease I (DNase I)	Thermo Scientific	EN0525
DL-Dithiothreitol	Sigma	D9779
dNTP Mix	Thermo Scientific	R0193
EDTA iron (III) sodium salt	Sigma-Aldrich	E6760
Ethidium bromide	Applichem	A1151
Ethylenediaminetatraaceticacid (EDTA)	Calbiochem	324503
GeneRuler 100 bp DNA Ladder	Thermo Scientific	SM0241
GeneRuler DNA Ladder Mix	Thermo Scientific	SM0332
Hoagland's No.2 basal salt mixture	Sigma-Aldrich	H2395
Isopropanol	Merck	1.09634
LB Agar	Sigma	L2897
LB Broth	Sigma	L302214
Magnesium chloride ($MgCl_2$)	Fluka	63063
Nuclease free water	Qiagen	129114

Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	P0662
Potassium sulfate (K_2SO_4)	Sigma-Aldrich	P0772
RevertAid H- M-MuLV RT	Thermo Scientific	EP0451
RNaseOUT	Life Technologies	10777-019
Sodium acetate (CH3COONa)	Sigma-Aldrich	S2889
Sodium hypochlorite (NaClO)	Sigma-Aldrich	425044
Taq DNA polymerase (recombinant)	Thermo Scientific	EP0401
TRI Reagent	Sigma	T9424
Trisbase	Sigma	T1503
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich	96495

APPENDIX B

Molecular Biology Kits

FirstChoice RLM-RACE Kit	Ambion	AM1700
Brilliant II SYBR Green QPCR Master Mix	Stratagene	600028
DH5α™ Competent Cells	Invitrogen	18265-017
FastStart Universal SYBR Green Master (ROX)	Roche	4913922001
GeneJET Gel Extraction Kit	Thermo Scientific	K0691
pGEM®-T Easy Vector System I	Promega	A1360
Plasmid Purification Kit	Qiagen	12123
SuperScript® III First-Strand Synthesis System	Invitrogen	18080-051
WizardH Genomic DNA Purification Kit	Promega	A1120

APPENDIX C

Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve 0T 032, TÜRKİYE
Balance:	Sartorius, BP221S, GERMANY
	Schimadzu, Libror EB-3 200 HU, JAPAN
Camera	Olympus C-7070, JAPAN
Centrifuge:	Microfuge 18 Centrifuge Beckman Coulter, USA
	Kendro Lab. Prod., Heraeus Multifuge 3S-R, GERMANY
	Kendro Lab. Prod., Sorvall RC5C Plus, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deepfreeze:	-20 ^{o}C Bosch, TURKEY
	-80 ^{o}C Thermo electron corporation, USA
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Labnet Gel XL Ultra V-2, USA
	Biogen Inc., USA
	Biorad Inc., USA
Fiter papers:	Whatman General Purpose Filtration Paper WHASE1141, Sigma, MO, USA
Gel Documentation:	Biorad Universal Hood II F1-F2 Fuses Type T2A, USA
	Biorad, UV-Transilluminator 2000, USA
Glassine crossing bags:	Focus Packaging & Design Ltd, North Lincolnshire, UK
Growth chamber:	Digitech DG12, Ankara, TURKEY
Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
Hydroponic tanks:	GroWell, UK
Ice Machine:	Scotsman Inc., AF20, USA
Incubator:	Innova 4330, USA
	Memmert, Modell 300, GERMANY

	Memmert, Modell 600, GERMANY
Laminar Flow:	Holten LaminAir Model 1.8 82034000, DENMARK
	Heraeus, Modell HS 12, GERMANY
Magnetic Stirrer:	VELP Scientifica, ITALY
Microarray:	Custom-made by LC Sciences, Houston, TX, USA
Microarray analysis:	GenePix 4000B Microarray Scanner, Axon Instruments, USA
	Array-Pro^{TM} Analyzer, Media Cybernetics, Silver Spring, MD, USA
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microscope	Olympus SZ61, JAPAN
	Olympus LG-PS2, JAPAN
Microwave digestion:	CEM-MARS Xpress system, USA
Microwave Oven:	Bosh, TÜRKİYE
Nitrogen tanks:	Linde Industrial Gases, TURKEY
Oven:	Memmert D06062 Modell 600, GERMANY
pH Meter:	WTW, pH540, GLP MultiCal, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
Real-Time PCR:	iCycler iQ Multi Color Real Time PCR Detection System, Bio-Rad, USA
Refrigerator:	+4 ^{o}C Bosh, TÜRKİYE
Sequencer:	Roche 454 GS FLX Sequencer, Basel, SWITZERLAND
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., InnovaTM 4330, USA
	New Brunswick Scientific Excells E24, USA
Spectrophotometer:	Amersham Biosciences Ultraspec 2100 pro, USA
	Nanodrop, ND-1000, USA
Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Thermocyler:	Eppendorf, Mastercycler Gradient, GERMANY
	Biorad Gradient Cycler DNA Engine, USA
Tissue Lyser:	Qiagen Retsch, USA
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK

Vortex Mixer:	VELP Scientifica 2X3, ITALY
Water bath:	Memmert, GERMANY

APPENDIX D

List of Primers Used in This Research

Barley microRNA study primers

Universal mature miRNA R:	5'GTGCAGGGTCCGAGGT3'
mature miR156 F:	5'GCGGCGGTGACAGAAGAGAGT3'
mature miR166 F:	5'TCGCTTCGGACCAGGCTTCA3'
mature miR171 F:	5'TTCCTTGATTGAGCCGCGCC3'
mature miR408 F:	5'GCGGCGGATGCACTGCCTCTTC3'
mature miR156 RT:	5'(stem-loop region)TGTGCT3'
mature miR166 RT:	5'(stem-loop region)GGGGAA3'
mature miR171 RT:	5'(stem-loop region)GATATT3'
mature miR408 RT:	5'(stem-loop region)GCCAGG3'
miR156 T (AV910992.1) F:	5'TCCCTTGAATGCCATGAACC3'
miR156 T (AV910992.1) R:	5'GGGATGCATTTATCTAGTCAGCA3'
miR171 T (BQ461013.1) F:	5'TGACCTCTACCTCACCCGAAA3'
miR171 T (BQ461013.1) R:	5'AGGAAGGCTGGAGCTGGAA3'
miR408 T (CB881899.1) F:	5'TTCAGGAGCACGCAGACGA3'
miR408 T (CB881899.1) R:	5'GAGTGAGTGTTTGATCGGTAGGA3'
RACE miR1436 T (CB862673.1):	5’GGGCAGGATCAAGAAGGTCAAA3’
RACE miR1436 T (CB862673.1):	5’ATCTTCCCTGCATCAGCTTTCC3’
RACE miR1436 T (CB862673.1):	5’GGAAGGGGTGGTGTCTTA3’
RACE miR156 (BE060620.2):	5’CAAGTCATTGTTGTCCGAGGTAGA3’

RACE miR156 T (BE060620.2): 5’GGGGATGTGTTCAGTCTGTTGG3’

RACE miR408 T (BU995745.1): 5’GATCGGTGGAGTCGAAATCAAA3’

RACE miR408 T (BU995745.1): 5’GCGCCGGACTTGTAGACCTT3’

stem-loop region:

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC

Bread wheat microRNA study primers

pre-miR169 F:	5’GTGGTAAGGGCTTCTTCTGG3’
pre-miR169 R:	5’CATATATACATGCAGAGATGTAAGTGT3’
pre-miR2118 F:	5’CCTGTGCAACTAGGGTCG3’
pre-miR2118 R:	5’TGGGAGGCATCGGGAAAT3’
pre-miR5070 F:	5’TCGAAGAACTAATTTAGTACTTTCCTA3’
pre-miR5070 R:	5’TGAAACCATCAGGGGTACAC3’
pre-miR5085 F:	5’AAGATAAAAAAAATGCCTTGTGAATAGA3’
pre-miR5085 R:	5’CAAGTTTCGATGAAAAGGGACATTT3’
pre-miR6220 F:	5’TACTACCTCCATCTCAAATCATAAGAC3’
pre-miR6220 R:	5’CCATTCCAACTCATCTATTTCCTC3’

Introgression study primers

XBARC77 F:	5’GCGTATTCTCCCTCGTTTCCAAGTCTG3’
XBARC77 R:	5’GTGGGAATTTCTTGGGAGTCTGTA3’

Normalization primers

BF474284 F:	5’CCATACTTGCATCCCCATCT3’
BF474284 R:	5’GTGTTGGATGAGCGCATTT3’
18s rRNA F:	5’GTGACGGGTGACGGAGAATT3’

18s rRNA R:	5’GACACTAATGCGCCCGGTAT3’

T: target, F: forward, R: reverse, RT: reverse transcription, RACE: RNA ligation mediated rapid amplification of cDNA ends, pre-miR: preliminary miRNA

APPENDIX E

Putative miRNA Cleavage Sites on Barley microRNA Targets Selected for Verification with RNA Ligation Mediated Rapid Amplification of Complementary DNA Ends

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Putative miR1436 target expressed sequence tag: CB862673.1 encoding for a hypothetical protein

AATATGGTACTCCCTCCGT↓CCCATAATATAAGGGCGTTTTCGACACTAGCA
TAATACATGAGTAACATAGAGCTATGAAGTACATAAAAGGAGTTTGTGCGG
TTGGCTTAGAGGTTTATAAATACCAGGACAACCGTACGCTGCTATATAGGC
AGAACTTTGGGCTATCACATTTGAAGTACATAAGCAAATAGTAAAGCACCA
GTTCGACGATTATTTTTAGTACTACAAAGGAGCAGGATCGAAGGGCGGCCC
CTTAGACGGCAGATGGATCAGTTGTTCTAACAACTCGTGGCGCTCTCTCCAT
GAGGACCAGTGGTTTTGGTCCGGCACAGTTGGCCTTCTTATTCTCCTTCTCTG
GGATAT**GGAAAGCTGATGCAGGGAAGAT**CTTAGAAAAGCCGGCAATACT
CTTGCAGATGAGCTTACCGGTTGCTGGTTCATCANGGGAGATCTCCTCCACT
GGGAGCCACACCATGAGCTCCCTGGGTCTTTATCCCTTT**GACCTTCTTGAT**
CCTGCCCTTCTCTGCAAAAGCAGTGNTCTCTGTGCCA**TAAGACACCACCCC**
TTCCTAAGGCCCTGGGNAGACGTGGGCCAACCC

Putative miR156 target expressed sequence tag: BE060620.2 encoding for teosinte glume architecture 1

GGAAACCGCAGCCGGATCCGATGAATTCTGCAAGTTTTATGACGAGTCAAC
AAGGAACAAGGTTTCCATCATTTCCAACTCCAAGACCGGAGCAAAACTGGC
CAGGGATCATTAAAACTGAGGAGAACCCCTATTACACACATCAGCTCCCTC
TAGGCATCAGTAACAGGCATCATTTTGGTGGCTCTACATCTACTTTCGCGAA
AGAAGGACGGAGATTTCCTTTCCTACAGGAGGGCGAAATAAACTTTGCCAC
CGGTGTGGCACTTGAGCCTGCAGTGTGCCAGCCGCTGCTCAAGACGGTAGC
TCCTCCCGAGAGCAGCAGCAGCAGCAGCAAGATGTTCTCCGATGGTCTGAC
TCCGGTGCTCGACTCGGACTGTGCTCTCTCT↓CTTCTGTCAGCTCCGGCAAAT
TCCTCCGGTATCGATGTTGGCCAGATGGT**CCAACAGACTGAACACATCCC**C
ATTNGCCCAACCTCTGTTCTNCAACCTGCAGCAATTCAGCAGCTCGTCCTGG
GTCTCGCGCACCCANGCTTNCACCGGCACCGTCTCAGCGACCCGATTTTNCT
TGCCCGTGGTGGAAAATGAGCAGCTGAACAATTG**TCTACCTCGGACAACA**
ATGACTTGAACTATCATGGGATTTTTCATGTTCGAAGCGAAAGCTTCTANA
ACGGCGCTTCGCATTNTTTGCNCTTCCCGGGGAAGAGTTTTCAGTGGCTGGT
CATG

Putative miR408 target expressed sequence tag: BU995745.1 encoding for chemocyanin

CGGACGAGGACTTGCTAGCTCCGGAGTGAGTGTTTGATCGGTAGGAGCTCC
TGAGGTAATTACTAGCAATGGCGACGCAGGGAAGA↓GGCAGTGCAGCCGTG
GTTGTGGGCGTGCTGCTCGTCTGCGTGCTCCTGAACGCAGCGGTGGCGGATG
CGGCGGTGTTCAACGTCGGCGACCGCGGCGGCTGGTCCTTCAACACCAACT
CCTGGCCCGCCGGCAAGCGCTTCAAGGCCGGCGACGTCCTAGTGTTCAAGT
ACGACGCGACGGCGCACGACGTGGTGGCGGTGAGCGCGGCAGGGTACAAG
GCCTGCGCCAAGCCGGCCAAGGGCGCC**AAGGTCTACAAGTCCGGCGC**CG
ACCGCGTCACCCTCGCCCGCGGCACAAACTACTTCATATGCAGCATCCCCGG
ACACTGCCAGTCCGGCATGAAGATCGCCGTCACCGCCGCCTAGATAGCCTC
GTCCATCGTCCATCATGCACGTAACTCGTACGCGCGACGTTACGTGACACGA
GAGTAATAATAATAACCAAAATAAAAGGCTGAGGGTATTATTCGTACGGTG
TGTGTACTG**TTTGATTTCGACTCCACCGATC**GTACTTGCTGTTGTATATGC
TGCCATGTGTAC

Primers are shown in bold. Putative mature miRNA binding sequence on target is underlined. Putative mature miRNA cleavage site on target is designated with ↓.

APPENDIX F

Preliminary microRNA Sequences of Predicted Barley microRNAs

microRNA	Sequence
miR1119	CUACGUGGCACGGCGCGAUGCUCAGUCAGCCAGUGGAGUGGAGUGGGGUGCUACCUUUUUACUACGAGUACGUACUAGGUGUGGGG
miR1120	CUACUCCCUCCUUCCCAUAUAUAAGAGUGUUUUUAAUACUACACUAGUGUGAAAAACAUUCUUAUAUUAUGGGACGGAGGAGUU
miR1121	AAGAGUGUUUAGAUCACUAACAUAGUGAUCUAAACGCUCUUACAGUGGCUGAAACUG
miR1122	AAUUAGUUGACUCAGAUUUGUCUAGAUUCGACAUGUUUUAGUGUUAGAUACAUCCGUAUCUAAACAAAUCUAAGACAACUAAUUUGGGAC
miR1126	UUUUUAAAGAUUCAACUAUGGACUACAUACGGAACAAAAUGAGUGAACUUACACUCUGAAUUAUGUCUAUAUAUAUAUCCAUAUGUAGCCCAUAGGGAAAUCUC
miR1139	GGAAGUAACUUAGACUAGUAACAUGUUACUAGUCUAUGUUACUACC
miR1436	GGCUUCCCUGUAUACAGACAGAGUCAUACUCCCUCCAUAAUGUAGUACGGUUUUUGACACUACUACAUUAUGGGACGGAGGGAGUACAUUG
miR1438	GGUACUCCCUCCGUUCCAAAAUACUUGUUGUACUUUUUGUUCAAAUUUGAACUAAAACCAUGACAAAUAUUUUUGGAACGGAGUGAGUA
miR156a	UGACAGAAGAGAGAGAGCACAGUCCGAGUCGAGCACCGGAGUCAGACCAUCGGAGAACAUCUUGCUGCUGCUGCU
miR159a	UUGUGGUUUGCAUGAUCGAGGAGCCGCUUCGAUCCCUCGCUGACCGCUGUUUGGAUUGAAGGGAGCUCUGCAUCUUGAUCUAUUACUA
miR160a	CCCGUGCGGUGACAGACUCGACUGCAGUUUGUUAAGGUGGAGAUCUGAUAAAGAUAUACCAAAUUGAGCAUGCCUGGCUCCCUGUAUGCCUGCAGGAGUGCCGUCCGGG
miR160b	UGCCUGGCUCCCUGUAUGCCUGCAGGAGAGUUGUUGUCCGAGUAACAGCAUAGGGGGCUGUUGUA
miR165	CGUCAUGGUUGUCGAGGGGAAUGACNCCGGGUCCNAAAGAGAGACNCUCGCAUGGCGUGCGCGUGGUGCGUUUCGGACCAGGCUUCAUUCCCCAUGACUCCAUCAUG
miR166m	GGGGAAUGACNCCGGGUCCNAAAGAGAGACNCUCGCAUGGCGUGCGCGUGGUGCGUUUCGGACCAGGCUUCAUUCCCC

miR166n	GGGGAAUGUUGUCUGGUCCGAGACCUAACACCGGGCGGAAUG GCGGAUUCAGCUGCAGCUAAGCAAGCUAGGUGGGGGGUUUCG GACCAGGCUUCAUUCCCC
miR168a	CGCCUCGGGCUCGCUUGGUGCAGAUCGGGACCCUCCGCCCGCC CCGACGGGCCGGAUCCCGCCUUGCACCAAGUGAAUCGGAGCC G
miR168b	UCGCUUGGUGCAGAUCGGGACCCUCCGCCCGCCCCGACGGGCC GGAUCCCGCCUUGCACCAAGUGA
miR171	GAUGUUGGCUCGACUCACUCAGACCACGCCGGAGGGAGCCAU CUGCGGCGGCGGUUCUGAUUGAGCCGUGCCAAUAUC
miR172a	AGGAUCUUGAUGAUGCUGCCGAGAAGGCUGUAAAAGCAUCGG UCAAAUGAUUA
miR172b	AGGAUCUUGAUGAUGCUGCCGAGAAGGCUGUGAAAGCAUCGG UCAAAUGAUUAAUGAUU
miR172c	AGGAUCUUGAUGAUGCUGCCGAGAAGGCUGUAAAAGCAUCGG UCAAAUGAUU
miR172d	AGGAUCUUGAUGAUGCUGCCGAGAAGGCUGUGAAAGCAUCGG UCAAAUGAUU
miR2055	GGGCUUUAUGGUGCUGGAUCCCUUAUAACUGAAGGUUUCCUU GGGAAGGGGGUUUUUUUAGGA
miR395a	AGCUGUCGGCAUGCGCCCAGGGUCAAUGAGCACGACGACAUC CAAAGGGAUCGCAUUGUCUCCAGCU
miR397	GUUGAGUGCAGCGUUGAUGAACCGUCCGGCCAUGGCCCGUCC GCCUCCACCGAGGCCGGAGCGGUUCACCGGCGCUGCACGCAAC
miR399g	GUACUGUAGGUAGGUUUUGCCAAAAGGAGAUUUGCCCCGAAC UAUCGGUAGGUUUGCCAAAGGAGAUUUGCCCCGAAG
miR408	GCUGCGUUCAGGAGCACGCAGACGAGCAGCACGCCCACAACC ACGGCUGCACUGCCUCUUCCCUGCGUCGC
miR444	AGGCACAAGUAGGACACCAACAUUACCUGCAAGCAAGACGCA AAAUUAAUAGAACAUCACCAUACUUGUGGCUUUCUUGCAAGU CGUGCAGUUGCUGCCUCAAGCUUGCUGCCU

APPENDIX G

List of Newly Identified Barley microRNAs and their Homologous microRNAs Detected in MicroPC

microRNA	Homologus microRNA(s) from MicroPC
miR1119	-
miR1120	tae-miR1120, osa-miR818e, osa-miR818d, osa-miR818c, osa-miR818b, osa-miR818a, osa-miR1436
miR1121	tae-miR1121
miR1122	tae-miR1122
miR1126	tae-miR1126
miR1139	-
miR1436	osa-miR1436, tae-miR1118
miR1438	osa-miR1439
miR156a	zma-miR156j, gma-miR156b, mtr-miR156, sbi-miR156d, osa-miR156k, ppt-miR529g, vvi-miR156h, ppt-miR529d, ppt-miR529c, ppt-miR529b
miR159a	tae-miR159a, tae-miR159b, zma-miR159b, zma-miR159a, sof-miR159d, sof-miR159b, sof-miR159a, sbi-miR159, osa-miR159b, osa-miR159a

miR160a	sly-miR160a, vvi-miR160f, vvi-miR160d, vvi-miR160c, tae-miR160, smo-miR160b, smo-miR160a, ppt-miR160f, ppt-miR160e, ppt-miR160a
miR160b	sly-miR160a, vvi-miR160f, vvi-miR160d, vvi-miR160c, tae-miR160, smo-miR160b, smo-miR160a, ppt-miR160f, ppt-miR160e, ppt-miR160a
miR165	sly-miR166b, sly-miR166a, vvi-miR166h, vvi-miR166g, vvi-miR166f, vvi-miR166e, vvi-miR166d, vvi-miR166c, bna-miR166d, bna-miR166c
miR166m	sly-miR166b, sly-miR166a, vvi-miR166h, vvi-miR166g, vvi-miR166f, vvi-miR166e, vvi-miR166d, vvi-miR166c, bna-miR166d, bna-miR166c
miR166n	sly-miR166b, sly-miR166a, vvi-miR166h, vvi-miR166g, vvi-miR166f, vvi-miR166e, vvi-miR166d, vvi-miR166c, bna-miR166d, bna-miR166c
miR168a	zma-miR168b, zma-miR168a, sof-miR168a, sbi-miR168, osa-miR168a, vvi-miR168, bna-miR168, ptc-miR168b, ptc-miR168a, gma-miR168
miR168b	zma-miR168b, zma-miR168a, sof-miR168a, sbi-miR168, osa-miR168a, vvi-miR168, bna-miR168, ptc-miR168b, ptc-miR168a, gma-miR168
miR171	sly-miR171a, tae-miR171, ptc-miR171i, ptc-miR171h, ptc-miR171g, ptc-miR171f, ptc-miR171e, zma-miR171i, zma-miR171e, zma-miR171j
miR172a	sly-miR172b, sly-miR172a, vvi-miR172d, vvi-miR172c, ptc-miR172h, ptc-miR172g, ptc-miR172f, ptc-miR172e, ptc-miR172d, ptc-miR172c
miR172b	sly-miR172b, sly-miR172a, vvi-miR172d, vvi-miR172c, ptc-miR172h, ptc-miR172g, ptc-miR172f, ptc-miR172e, ptc-miR172d, ptc-miR172c
miR172c	sly-miR172b, sly-miR172a, vvi-miR172d, vvi-miR172c, ptc-miR172h, ptc-miR172g, ptc-miR172f, ptc-miR172e, ptc-miR172d, ptc-miR172c
miR172d	sly-miR172b, sly-miR172a, vvi-miR172d, vvi-miR172c, ptc-miR172h, ptc-miR172g, ptc-miR172f, ptc-miR172e, ptc-miR172d, ptc-miR172c
miR2055	-
miR395a	vvi-miR393a, gma-miR393, vvi-miR393b, bna-miR393, ptc-miR393d, ptc-miR393c, ptc-miR393b, ptc-miR393a, zma-miR393, mtr-miR393
miR397	sly-miR397, vvi-miR397b, vvi-miR397a, bna-miR397b, bna-miR397a, ptc-miR397b, ptc-miR397a, osa-miR397b, osa-miR397a, ath-miR397a

miR399g	ptc-miR399a, ath-miR399d, vvi-miR399g, vvi-miR399e, bna-miR399, ptc-miR399c, ptc-miR399b, mtr-miR399e, mtr-miR399a, mtr-miR399c
miR408	tae-miR408, zma-miR408, sof-miR408d, sof-miR408c, sof-miR408b, sof-miR408a, osa-miR408, vvi-miR408, smo-miR408, ppt-miR408b
miR444	osa-miR444e, osa-miR444d.1, osa-miR444d.2, tae-miR444, osa-miR444a.1, osa-miR444a.2, osa-miR444d.3, osa-miR444f, osa-miR444c.1, osa-miR444b.1

tae: *Triticum aestivum*, osa: *Oryza sativa*, zma: *Zea mays*, gma: *Glycine max*, mtr: *Medicago truncatula*, sbi: *Sorghum bicolor*, ppt: *Physcomitrella patens*, vvi: *Vitis vinifera*, sof: *Saccharum officinarum*, sly: *Solanum lycopersicum*, smo: *Selaginella moellendorffii*, ptc: *Populus trichocarpa*, bna: *Brassica napus*, ath: *Arabidopsis thaliana*

APPENDIX H

Predicted Targets of Putative Barley microRNAs

microRNA	Target sequence	Target protein
miR1119	GH228802.1, GH223038.1, GH223037.1, FD527818.1, FD525832.1, FD525157.1, BY874148.1, BY864489.1, BY873820.1, BY873695.1, BY872818.1, BY861857.1, BY870666.1, BY860649.1, CB881890.1, CB880638.1, CB880289.1, CB880289.1, CB876132.1, CB875296.1, CB874935.1, CB874734.1, CB874459.1, CB874191.1, CB874075.1, CB872600.1, CB872453.1, CB872284.1, CB872255.1, CB872108.1, CB871762.1, CB871755.1, CB870604.1, CB868554.1, CB868369.1, CB867081.1, CB866608.1, CB859747.1, CB858914.1, CB858768.1, CB858345.1, CB858181.1, CA029265.1, CA028720.1, CA027359.1, CA027063.1, CA026736.1, CA025269.1, CA020594.1, CA018933.1, CA018263.1, CA017073.1, A016083.1, BU994188.1, BU993977.1, BU986680.1, BU975947.1, BU975470.1, BU973972.1, BU973892.1, BU973607.1, BU971666.1, BU970318.1, BQ761269.1, BQ761024.1, BQ760313.1, BQ756701.1, BQ756674.1, BI776713.2, BI776712.2, BI776941.2, BM441507.2, BM372872.2, BM372821.2, BM096529.2, BM096491.2, BI781077.2, BM443814.2, BQ665864.1, BQ661979.1, BQ660599.1, BQ660575.1, BQ134689.1, BQ467836.1, BQ462148.1, AJ486203.1, AJ486202.1, BM445267.1, BM444358.1, BM444122.1, BM443806.1, BM443638.1, BG343309.2, BG342907.2, BI960270.1, BI959664.1, BI959039.1, BI946902.1, BI776735.1, BG417782.1	leucine rich repeat-containing protein, unknown protein
miR1120	EX584378.1, FD519329.1, EX584378.1, BU974091.1, BQ766259.1, BJ460470.1, AJ461536.1	unknown, csAtPR5, proteinase inhibitor, acid phosphatase/vanadium-dependent haloperoxidase related

miR1121	BY867242.1, DN186686.1, BQ761197.1, BQ656484.1, BQ458760.1, AV928265.1, GH208317.1, FC555790.1, EX591441.1, EX591437.1, EX587628.1, EX587623.1, DN187056.1, CK569040.1, CK568591.1, CK565957.1, U970654.1, BJ461877.1, BJ448740.1, AV944995.1, BI956127.1, AV836141.1, BE216561.2, EX601113.1, EX601112.1, BY848300.1, BY845018.1, DN188870.1, AJ484964.1, AJ484963.1, BJ455329.1, BJ447578.1, AV927890.1, AV918531.1, AV912245.1, AV910077.1	cell cycle control protein-related, coiled-coil domain-containing protein 94, pti1 kinase-like protein, serine/threonine protein kinase,
miR1122	BY876819.1, FD518678.1, DN157625.1, EX585467.1, EX585466.1, CX627768.1, BI953109.1	pp70 ribosomal protein S6 kinase, NBS-LRR resistance protein RGH2, zinc finger (C3HC4-type RING finger),
miR1126	CX629047.1, CA012080.1, AJ469590.1, CX631396.1, GH209357.1, EX594051.1, EX581233.1, BY875458.1	high-affinity potassium transporter, B70; transcription factor,
miR1139	EX582279.1, EX582278.1, BQ464633.1	poly(A) polymerase-like protein
miR1436	DN159362, BM100733.2, BJ485923.1, BJ485923.1, BJ485913.1, GH219034.1, FD519329.1, EX573766.1, EX595547.1, EX595552.1, EX600769.1, BY877744.1, BY874438.1, BY865147.1, CV064340.1, CK569835.1, CD663278.1, CD057247.1, CB863041.1, CB862673.1, CA010088.1, BM371881.2, BJ482549.1, BJ468182.1, AV834592.1, AW983316.2, GH213790.1, FD521311.1, EX581160.1, DN187130.1, DN184585.1, DN180754.1, CX629150.1, CB877969.1, CA010562.1, BU996201.1, BU977032.1, BU968160.1, BQ662944.1, BQ661883.1, BQ661628.1, BQ657747.1, BQ467319.1, BJ486097.1, AV834234.1, AL509815.1, AL503060.1, AL500558.1, GH217963.1, GH212963.1, EX575535.1, EX583817.1, EX592251.1, EX592250.1, EX593248.1, EX591321.1, EX591500.1, EX583713.1, EX583708.1, EX593880.1, EX580539.1, EX580538.1, BY874640.1, CB871794.1, CB867028.1, BM443538.2, BG344337.1	Avr9/Cf-9 rapidly elicited protein, expressed protein, hypothetical protein OsI_34102, hypothetical protein SORBIDRAFT_05g026800, bZIP transcription factor bZIP109, NADH dehydrogenase

miR1438	BF256174.1, BU984969.1, BJ473266.1, BJ472225.1, BJ471375.1, BJ470543.1, GH218489.1, FD528314.1, FD528241.1, FD520850.1, FD520292.1, FD518968.1, EX595629.1, EX595574.1, EX595570.1, EX575273.1, EX587834.1, EX592026.1, EX592021.1, EX585864.1, EX583410.1, EX572525.1, EX572520.1, EX597806.1, EX597805.1, EX585782.1, EX585781.1, EX583409.1, BY864230.1, BY864094.1, BY863662.1, BY862007.1, BY859316.1, BY858575.1, BY877400.1, BY865328.1, BY874243.1, DN185957.1, DN159007.1, DN157326.1, CK568443.1, CD664034.1, CB879598.1, CB862607.1, BU988058.1, BU981792.1, BU974046.1, BU971496.1, BU968784.1, BM377451.2, BQ661000.1, BQ658389.1, BQ658360.1, BJ479954.1, BJ466768.1, BJ464326.1, AJ463403.1, AJ434654.1, AV930760.1, AV923231.1, BI950824.1, BF625612.3, BF253848.2	AAA-type ATPase family protein, unknown protein, DNA helicase-like,
miR156a	FD528446.1, FD526024.1, FD526024.1, FD527301.1, EX597081.1, EX597080.1, EX598089.1, CA032492.1, CA020690.1, BJ478354.1, BF258419.2, BE060620.2, GH228608.1, BQ665502.1, BJ485673.1, AV910992.1, AV909109.1, BQ757841.1, BG300360.1, EX576980.1, EX593949.1, CB858958.1	teosinte glume architecture 1, squamosa promoter-binding protein, SBP-domain protein 6,9, squamosa promoter binding protein-like 4
miR159a	AJ475696.1, BJ448559.1, CA006699.1, AJ480356.1, AJ480355.1	transcription factor GAMyb Myb33
miR160a	GH209508.1, BU986263.1, BJ472971.1, BJ472971.1, BJ471957.1, BJ471544.1, BJ469735.1, BJ469445.1, AV934494.1, AV934488.1, AV933114.1, AV932125.1, BF622299.2, CA002787.1, BF622307.1	auxin response factor 16, auxin response factor 10
miR165	BY841849.1	-
miR171	BQ461013.1	SCL6 transcription factor

miR172a	BY867399.1, DN185554.1, CX627187.1, CV062201.1, CV060423.1, CV059650.1, CB872997.1, CB872642.1, CB868609.1, CB864455.1, BJ553068.1, BJ552631.1, BJ551880.1, BJ550102.1, BJ549954.1, BJ549884.1, BJ549797.1, BJ549795.1, BJ549763.1, BJ549614.1, BJ546100.1, BJ546031.1, BU991171.1, BQ763181.1, BQ760907.1, AJ436477.1, AV921360.1, BF261929.2, FD525656.1, FD519858.1, CB883643.1, CB859285.1, CB858710.1, CA031544.1, BU977981.1, BU977689.1, BU977520.1, BQ468903.1, AL511903.1, AL501298.1, AL499992.1, GH206429.1	succinyl-CoA ligase beta-chain
miR2055	GH219150.1, FD522016.1, FD519704.1, BY877030.1, CB863733.1, CB862831.1, BQ660241.1, BQ657284.1, BJ467047.1, AJ434678.1, AV921187.1, AV919340.1, BM100874.1, AL511307.1,	minichromosome maintenance protein, ATPase
miR395a	CV055207.1, CB860441.1, CA004635.1, BU973213.1, BJ461212.1, BJ453671.1, AJ432418.1, BE603282.2, BI953281.1	transport inhibitor response, AFB2 (AUXIN SIGNALING F-BOX 2), auxin-responsive factor TIR1-like protein,
miR397	FD518508.1, CA007960.1, CA005607.1, BU966899.1, BU966762.1, BQ665274.1, BQ665274.1, BQ461815.1, BM097863.1, BG415888.2, DN179783.1, BU973559.1, BQ663574.1, BI960614.1, BI960367.1, BI960433.1, BI957285.1, BI957118.1, BI956799.1	vacuolar protein sorting-associated protein, laccase LAC6-2
miR408	CB881899.1, BU995745.1, BU995231.1, BU976391.1, BQ768355.1, BM372993.2, BQ468842.1, BM817241.1, BG344510.1, GH228273.1, CA021788.1, BU995189.1, BU994743.1, BI958939.1, BI957261.1	chemocyanin, blue copper-binding protein
miR444	GH228935.1, CA019616.1, CA008118.1, CA006693.1, BQ765702.1, BQ765438.1, BQ754066.1, AL504006.1, CX630154.1, DN188095.1, GH224063.1	putative transcription factor MADS27, cryptochrome 1b

Target sequence: GeneBank accession

APPENDIX I

microRNA Cleaved Barley Sequences Identified by RNA Ligation Mediated Rapid Amplification of Complementary DNA ends

sequence 1

5'GGTCCCTGGGAAGAGACTTCACAATCTGGCTATGGGTTGGCATCCCCAAC
AGACTGAACACATCCC3'

sequence 2

5'GGGGGTCGCAGTGACCAGGCCCGGGCGACTGTTTACCAAAAACACAGGTC
TCCGCAAAGTCGTAAGACCATGTATGGGGGCTGACGCCTGCCCAGTGCCGG
AAGGTCTACAAGTCCGGCGC3'

sequence 3

5'TACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCA
TGCATGTGCAAGTATGAACCAATTTGACCTTCTTGATCCTGCCC3'

sequence 4

5'AAGCCATGCATGTGCAAGTATGAACCAACAGACTGAACACATCCC3'

sequence 5

5'TCTCATGGAGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCTTA
ACACATGCAAGTCGAACGGGAAGTGGTGTTTCCAGTGGCGAACGGGTGAGT
AACGCGTAAGAACCTGCCCTTGGGAGGGGAACAACAACTGGAAACGGTTGC
TAATACCCCGTAGGCTGAGGAGCAAAAGGAGAAATCCGCCCAAGGAGGGG
CTCGCGTCTGATTAGCGAGTTGGTGAGGCAATAGCTTACCAAGGCGATGAT
CAGTAGCTGGTCCGAGAGGATGATCAGCCACACTGGGGCTGAGACACGGCC
CAGACTCCTACGGGAGGCANCAGTGGGGAATTTTCCGCAATGGGCGAAAGC
CTGACGGANCAATGCCGCGTGGAGGTGGAAGGCCTACGGGTCGTCAACTTC
TTTTCTCGGAGAAGAAACAATGACGGTATCTGAGGAATAAGCATCGGCTAA
CTCTGTGCCAGCAGCCGCGGTAAGACAGANGATGCAAGCGTTATCCGGGAA
AGCTGATGCAGGGAAGAT3'

sequence 6

5'AACCGTAGCTCCTCACCTACTCACCCATGGATGGATGGATGGATGGATGG
GGACGGGGCAATGACGCGGTGGATCCACAGCCGCAACCACCTTCTCTCGAT
GGGGACCTCGACACCGACGAACACCACTTCTACCTCGGACAACAATGACTT
3'

sequence 7

5'TCAAAAGAGGAAAGGCTTGCGGTGGATACCTAGGCACCCAGAGACGAGG
AAGGTCTACAAGTCCGGCG3'

APPENDIX J

Putative microRNAs that Cleave Sequences Obtained by Rapid Amplification of Complementary DNA ends and their Known Targets

Sequence	microRNA	Known targets
1.	hvu-miRNA165	HD-Zip transcription factors including Phabulosa (PHB) and Phavoluta (PHV)
	hvu-miRNA166	HD-Zip transcription factors including Phabulosa (PHB) and Phavoluta (PHV)
	ath-miRNA408	plantacyanin, copper ion binding
	mtr-miRNA1510	resistance genes, aminophospoholipid ATPase, ATP binding cassette transporter, brassionosteroid receptor
2.	_	_
3.	gma-miRNA1511	GRAS family transcription factor
	ppt-miRNA900-5p	similar to AP endonuclease/reverse transcriptase
		branched-chain aminotransferase 4, similar to microfilarial sheath protein SHP3 precursor
4.	ppt-miRNA538	similar to AP endonuclease/reverse transcriptase,
		serine/threonine protein phosphatase 2A, similar to microfilarial sheath protein SHP3 precursor
5.	hvu-miRNA156	squamosa-promoter binding protein (SBP) box
	ppt-miRNA1214	beta,beta-carotene 9,10-dioxygenase
6.	hvu-miRNA2055	nucleic acid binding protein, Zn-finger, RanBP-type containing protein, stripe rust resistance protein,
		ATP binding protein, retrotransposon protein, protein neuralized, laccase
7.	hvu-miRNA171	GRAS domain or SCARECROW-like proteins
	hvu-miRNA172	Apetala 2 (AP2) proteins
	hvu-miRNA397	laccases and beta-6 tubulin
	hvu-miRNA159	MYB proteins

	ath-miRNA853	retroposon, Ty3-gypsy class

Known targets are listed from miRBase (version 13, March 2009)

hvu: *Hordeum vulgare*, ath: *Arabidopsis thaliana*, mtr: *Medicago truncatula*, gma: *Glycine max*, ppt: *Physcomitrella patens*

APPENDIX K

Probes Detecting Homologous microRNAs in Wild Emmer Wheat Control Tissues

ath-miR156a	mtr-miR395a	**osa-miR50-5p**	ptc-miR474b
ath-miR156g	mtr-miR395b	osa-miR535	ptc-miR474c
ath-miR156h	mtr-miR395g	ppt-miR1034	sbi-miR156e
ath-miR157a	mtr-miR395h	ppt-miR1066	sbi-miR164c
ath-miR157d	mtr-miR395p	ppt-miR160b	sbi-miR166a
ath-miR159a	mtr-miR399b	ppt-miR160c	sbi-miR171f
ath-miR159b	mtr-miR399d	ppt-miR166j	sly-miR319
ath-miR159c	osa-miR1318	ppt-miR166m	sly-miR395a
ath-miR160a	osa-miR1427	ppt-miR167	sly-miR397
ath-miR164a	osa-miR1432	ppt-miR390c	smo-miR1082a
ath-miR164c	osa-miR1436	ppt-miR395	smo-miR1082b
ath-miR165a	osa-miR156l	ppt-miR477a-3p	smo-miR1091
ath-miR166a	osa-miR159a	**ppt-miR536a**	smo-miR1105
ath-miR167a	osa-miR159c	ppt-miR893	smo-miR156b
ath-miR167c	osa-miR159d	ppt-miR894	smo-miR156c
ath-miR167d	osa-miR159e	ppt-miR896	smo-miR156d
ath-miR168a	osa-miR159f	pta-miR1310	**smo-miR171b**
ath-miR169a	osa-miR160e	pta-miR159a	sof-miR159e
ath-miR169b	osa-miR164c	pta-miR159b	sof-miR168b
ath-miR169d	osa-miR164d	pta-miR159c	tae-miR1118
ath-miR169h	osa-miR164e	pta-miR166c	tae-miR1125
ath-miR171a	osa-miR166e	pta-miR319	tae-miR1132
ath-miR171b	osa-miR166i	**ptc-miR1447**	tae-miR1134
ath-miR172e	osa-miR166k	ptc-miR1450	tae-miR399
ath-miR319a	osa-miR166m	ptc-miR156k	vvi-miR156e
ath-miR319c	osa-miR168a	ptc-miR159d	vvi-miR156h
ath-miR393a	osa-miR168b	ptc-miR159e	vvi-miR159a
ath-miR395a	osa-miR169e	ptc-miR159f	vvi-miR166a
ath-miR395b	osa-miR169n	ptc-miR160e	vvi-miR166b
ath-miR397a	osa-miR171h	ptc-miR160g	vvi-miR166c
ath-miR398a	osa-miR1846a-3p	ptc-miR160h	vvi-miR166d
ath-miR398b	osa-miR1846a-5p	ptc-miR164f	vvi-miR167c
ath-miR399b	osa-miR1846d-3p	ptc-miR166n	vvi-miR169l
ath-miR399d	osa-miR1858a	ptc-miR166p	vvi-miR169m
ath-miR404	osa-miR1861n	ptc-miR167f	vvi-miR169o
ath-miR853	osa-miR1867	ptc-miR167h	vvi-miR171a
ath-miR854a	osa-miR1883a	ptc-miR169o	vvi-miR171g
bna-miR156a	osa-miR319a	ptc-miR169q	vvi-miR171h
bna-miR167a	osa-miR393b	ptc-miR169s	vvi-miR171i
bna-miR169c	osa-miR395a	ptc-miR169t	vvi-miR319b
bna-miR171g	osa-miR395b	ptc-miR169v	vvi-miR319e
bna-miR393	osa-miR395c	ptc-miR169x	vvi-miR319g
ghr-miR156c	osa-miR395f	ptc-miR171c	vvi-miR396a
gma-miR1532	osa-miR395o	ptc-miR171e	vvi-miR396b
gma-miR156b	osa-miR395t	ptc-miR171k	vvi-miR399i
gma-miR159b	osa-miR396d	ptc-miR319e	vvi-miR535a
gma-miR159c	osa-miR397b	ptc-miR319i	zma-miR171b
gma-miR167c	osa-miR415	ptc-miR395a	zma-miR171c
gma-miR169c	osa-miR444a.1	ptc-miR397b	zma-miR171f
gma-miR171a	osa-miR444a.2	ptc-miR398b	
gma-miR319a	osa-miR444b.1	ptc-miR399f	
gma-miR319c	osa-miR528	ptc-miR474a	

	only in leaf
	only in root
	in both control tissues
miR	in controls but not stressed
refer to miRBase for microRNA nomenclature	

APPENDIX L

Probes Detecting Homologous microRNAs in Wild Emmer Wheat Dehydrated Tissues

	4 hour stressed leaf		4 hour stress, both tissues	***bold & italic***	in all tissues and timepoints
	8 hour stressed leaf		8 hour stress, both tissues	miR	only in stress samples, not in controls
	4 hour stressed root		leaf only, both timepoints	refer to miRBase for microRNA nomenclature	
	8 hour stressed root		root only, both timepoints		

ath-miR156a	ath-miR778	***gma-miR319a***	***osa-miR1858a***	ppt-miR1029	ppt-miR902c-3p	ptc-miR397b	***sof-miR159e***
ath-miR156g	ath-miR779.1	gma-miR319c	osa-miR1861g	ppt-miR1030a	ppt-miR902h-3p	***ptc-miR398b***	***sof-miR168b***
ath-miR156h	ath-miR779.2	gma-miR390b	osa-miR1861n	ppt-miR1030i	ppt-miR903	***ptc-miR399f***	sof-miR408e
ath-miR157a	ath-miR780.1	gma-miR482	***osa-miR1867***	ppt-miR1031a	ppt-miR904a	ptc-miR399h	***tae-miR1118***
ath-miR157d	ath-miR780.2	mtr-miR171	osa-miR1868	ppt-miR1032	pta-miR1309	ptc-miR399i	tae-miR1124
ath-miR158a	ath-miR781	***mtr-miR395a***	osa-miR1870	ppt-miR1033a	pta-miR1310	ptc-miR399j	***tae-miR1125***
ath-miR159a	ath-miR782	***mtr-miR395b***	osa-miR1875	ppt-miR1034	pta-miR1311	ptc-miR399l	tae-miR1128
ath-miR159b	ath-miR824	***mtr-miR395g***	osa-miR1876	ppt-miR1035	pta-miR1312	***ptc-miR472a***	tae-miR1129
ath-miR159c	ath-miR826	***mtr-miR395h***	osa-miR1877	ppt-miR1041	pta-miR1314	***ptc-miR472b***	tae-miR1132
ath-miR160a	ath-miR827	***mtr-miR395p***	osa-miR1879	ppt-miR1043-3p	pta-miR1315	ptc-miR473a	tae-miR1133
ath-miR161.1	ath-miR829.1	mtr-miR399b	osa-miR1881	ppt-miR1047-3p	pta-miR1316	ptc-miR473b	***tae-miR1134***
ath-miR163	ath-miR829.2	mtr-miR399d	osa-miR1883a	ppt-miR1050	pta-miR156a	ptc-miR474a	tae-miR399
ath-miR164a	ath-miR830	osa-miR1317	***osa-miR319a***	ppt-miR1053-3p	pta-miR156b	***ptc-miR474b***	***vvi-miR156e***
ath-miR164c	ath-miR832-5p	***osa-miR1318***	osa-miR393b	ppt-miR1054	***pta-miR159a***	***ptc-miR474c***	***vvi-miR156h***
ath-miR165a	ath-miR833-3p	osa-miR1423	***osa-miR395a***	ppt-miR1055	***pta-miR159b***	ptc-miR475a	***vvi-miR159a***
ath-miR166a	***ath-miR839***	osa-miR1423b	***osa-miR395b***	ppt-miR1056	***pta-miR159c***	ptc-miR475d	vvi-miR166a
ath-miR167a	ath-miR841	osa-miR1425	osa-miR395c	ppt-miR1057	pta-miR166c	ptc-miR478d	***vvi-miR166b***
ath-miR167c	ath-miR843	osa-miR1427	osa-miR395f	ppt-miR1058	***pta-miR319***	Ptc-miR482.1	***vvi-miR166c***
ath-miR167d	ath-miR846	osa-miR1431	osa-miR395o	***ppt-miR1066***	***pta-miR398***	Ptc-miR482.2	***vvi-miR166d***
ath-miR168a	ath-miR847	***osa-miR1432***	osa-miR395t	ppt-miR1068	pta-miR482a	ptc-miR530b	***vvi-miR167c***
ath-miR169a	ath-miR852	osa-miR1433	osa-miR395u	ppt-miR1069-3p	pta-miR482d	***sbi-miR156e***	vvi-miR169b
ath-miR169b	***ath-miR854a***	osa-miR1435	osa-miR395v	ppt-miR1076-3p	pta-miR946a*	***sbi-miR164c***	vvi-miR169i
ath-miR169d	ath-miR855	***osa-miR1436***	***osa-miR396d***	ppt-miR1077-5p	pta-miR949	***sbi-miR166a***	***vvi-miR169l***
ath-miR169h	ath-miR856	osa-miR1437	osa-miR397b	ppt-miR1223a	pta-miR952a	sbi-miR171f	vvi-miR169m
ath-miR170	ath-miR858	osa-miR1439	osa-miR399h	ppt-miR1223b	ptc-miR1448	Sly-miR171d	vvi-miR169o
ath-miR171a	ath-miR862-3p	osa-miR1440	osa-miR399k	ppt-miR1223d	***ptc-miR1450***	Sly-miR1916	vvi-miR169r
ath-miR171b	ath-miR863-3p	osa-miR1441	osa-miR408	ppt-miR1223e	***ptc-miR156k***	Sly-miR1919a	vvi-miR169t
ath-miR172e	ath-miR864-5p	osa-miR1442	osa-miR413	ppt-miR1223h	***ptc-miR159d***	***sly-miR319***	vvi-miR169v
ath-miR319a	ath-miR865-3p	***osa-miR156l***	***osa-miR415***	ppt-miR1223j	***ptc-miR159e***	***sly-miR395a***	vvi-miR169y
ath-miR319c	bna-miR1140a	***osa-miR159a***	osa-miR438	***ppt-miR160b***	***ptc-miR159f***	***sly-miR397***	vvi-miR171a
ath-miR390a	bna-miR1140b	***osa-miR159c***	***osa-miR444a.1***	***ppt-miR160c***	ptc-miR160e	smo-miR1080	vvi-miR171b
ath-miR393a	***bna-miR156a***	***osa-miR159d***	***osa-miR444a.2***	ppt-miR160d	***ptc-miR160g***	smo-miR1082a	vvi-miR171f
ath-miR394a	bna-miR161	***osa-miR159e***	***osa-miR444b.1***	ppt-miR160h	***ptc-miR160h***	smo-miR1082b	vvi-miR171g
ath-miR395a	***bna-miR167a***	***osa-miR159f***	osa-miR444b.2	***ppt-miR166j***	***ptc-miR164f***	smo-miR1083	vvi-miR171h
ath-miR395b	bna-miR169c	***osa-miR160e***	osa-miR444f	ppt-miR166m	***ptc-miR166n***	smo-miR1084	vvi-miR171i
ath-miR396a	bna-miR169g	***osa-miR164c***	***osa-miR528***	***ppt-miR167***	***ptc-miR166p***	smo-miR1086	vvi-miR172b
ath-miR396b	bna-miR169m	***osa-miR164d***	osa-miR529a	ppt-miR390c	***ptc-miR167f***	Smo-miR1088-5p	***vvi-miR319b***
ath-miR397a	bna-miR171g	***osa-miR164e***	osa-miR530-3p	***ppt-miR395***	***ptc-miR167h***	smo-miR1091	***vvi-miR319e***
ath-miR398a	bna-miR393	***osa-miR166e***	osa-miR530-5p	ppt-miR408b	ptc-miR169aa	smo-miR1092	***vvi-miR319g***
ath-miR398b	bna-miR397a	osa-miR166i	osa-miR531	ppt-miR477a-3p	ptc-miR169ab	smo-miR1093	vvi-miR395n
ath-miR399a	***ghr-miR156c***	***osa-miR166k***	***osa-miR531b***	ppt-miR529a	***ptc-miR169o***	smo-miR1097	***vvi-miR396a***
ath-miR399b	gma-miR1516	***osa-miR166m***	osa-miR535	ppt-miR529d	ptc-miR169q	smo-miR1098	***vvi-miR396b***
ath-miR399d	gma-miR1517	***osa-miR168a***	osa-miR810a	***ppt-miR529e***	***ptc-miR169s***	smo-miR1100	vvi-miR399g
ath-miR399e	gma-miR1519	***osa-miR168b***	osa-miR814a	ppt-miR529g	ptc-miR169t	Smo-miR1101-3p	vvi-miR399i
ath-miR399f	gma-miR1520a	***osa-miR169e***	osa-miR815a	ppt-miR533a	ptc-miR169u	smo-miR1102	***vvi-miR535a***
ath-miR400	gma-miR1522	osa-miR169n	osa-miR820a	ppt-miR533a*	ptc-miR169v	***smo-miR1103-5p***	vvi-miR845c
ath-miR401	gma-miR1524	osa-miR171h	osa-miR821a	ppt-miR533b-5p	ptc-miR169x	smo-miR1105	zma-miR169d
ath-miR402	gma-miR1527	osa-miR1846a-3p	osa-miR827	ppt-miR533e	***ptc-miR171c***	***smo-miR1108***	zma-miR169e
ath-miR404	gma-miR1532	***osa-miR1846a-5p***	ppt-miR1023a-3p	ppt-miR536f	***ptc-miR171e***	smo-miR1110	zma-miR171a
ath-miR413	***gma-miR156b***	osa-miR1846c-3p	ppt-miR1023a-5p	ppt-miR893	ptc-miR171j	***smo-miR156b***	***zma-miR171b***
ath-miR447a	***gma-miR159b***	osa-miR1846d-3p	ppt-miR1023c-3p	***ppt-miR894***	ptc-miR171k	***smo-miR156c***	***zma-miR171c***
ath-miR472	***gma-miR159c***	osa-miR1847.1	ppt-miR1023c-5p	***ppt-miR896***	***ptc-miR319e***	***smo-miR156d***	***zma-miR171f***
ath-miR774	***gma-miR167c***	osa-miR1847.2	ppt-miR1024a	ppt-miR900-3p	***ptc-miR319i***	smo-miR171a	zma-miR399b
ath-miR775	***gma-miR169c***	osa-miR1848	ppt-miR1026a	ppt-miR900-5p	ptc-miR395a	smo-miR171c	
ath-miR776	gma-miR171a	osa-miR1850	ppt-miR1028c-5p	ppt-miR902a-3p	ptc-miR396f	smo-miR319	

APPENDIX M

Characteristics of Selected Preliminary microRNAs Predicted from the Long Arm of Bread Wheat Chromosome 5D

New bread wheat microRNA	Bread wheat mature microRNA sequence	Accession	Homologous miRNA	Location	ΔG (kcal/mol)	LP	LM	GC (%)	MFEI
miR1117	UAGUACCGGUUCGUGGCACGAACC	HRFOW6E02IYSKI	tae-miR1117	5'	-104.8	152	24	57.23684	1.204598
miR1118	CACUACAUUAUGGAAUGGAGGGA	HRFOW6E02H3OY4	tae-miR1118	3'	-127.2	291	23	32.98969	1.325
miR1120	ACGUUCUUAUAUUAUGGGACGGAG	HRFOW6E02I6LX0	tae-miR1120	3'	-37.8	110	24	38.18182	0.9
miR1121	UAUAUUGAUCUAAACGCUCUUA	HRFOW6E02HC5B0	tae-miR1121	3'	-29.2	96	22	32.29167	0.941935
miR1122	UAGAUACAUCCGUAUUUAGA	HRFOW6E02H7L52	bdi-miR1122	3'	-50	93	20	26.88172	2
miR1123	CACGUGAGACCUGGUCUCAUAGA	HJKAX1S02HNBZ4	tae-miR1123	3'	-161.5	252	23	49.60317	1.292
miR1125	AACCAACGAGACCGACUGCGGCGG	HRFOW6E02JQX8D	tae-miR1125	5'	-87.4	153	24	37.2549	1.533333
miR1127	AACUACUCCCUCCGUCCCAUA	HRFOW6E02JBR5E	bdi-miR1127	5'	-71.1	130	21	40	1.367308
miR1128	UACUACUCCCUCCGUCCGAAA	HRFOW6E02G37AT	tae-miR1128	5'	-76.3	164	21	40.85366	1.138806
miR1130	CCUCCGUCUCAUAAUGUAAGACG	HRFOW6E02IEDIM	tae-miR1130	5'	-65.7	117	23	42.73504	1.314
miR1131	UAGUACCGGUUCGUGGCUAACC	HRFOW6E02JH3PY	tae-miR1131	5'	-63	97	22	56.70103	1.145455
miR1133	GUUAUACUCCCUCCGUCCGAAA	HRFOW6E02FKQJJ	tae-miR1133	5'	-53.3	156	22	37.82051	0.90339
miR1135	CUGCGACAAGUAAUUCCGAACGGA	HRFOW6E02I89BW	tae-miR1135	3'	-80.5	131	24	41.98473	1.463636
miR1136	UUGUCGCAGGUAUGGAUGUAUCUA	HRFOW6E02G8ADT	tae-miR1136	5'	-69.1	105	24	40.95238	1.606977
miR1137	UAGUACAAAGUUGAGUCAUC	HRFOW6E02F1AHM	tae-miR1137	3'	-61.2	93	20	36.55914	1.8
miR1139	AGAGUAACUUACACUAGUAACA	HRFOW6E02IN3KE	tae-miR1139	5'	-31.7	90	22	36.66667	0.960606
miR1436	ACAUUAUGGGACGGAGGGAGU	HRFOW6E02JT21O	hvu-miR1436	3'	-53.8	122	21	33.60656	1.312195
miR1439	UUUUGGAACGGAGUGAGUAUU	HRFOW6E02IP2QZ	osa-miR1439	3'	-64.7	130	21	37.69231	1.320408
miR160	GCCUGGCUCCCUGUAUGCCAC	HJKAX1S02HK6ZI	csi-miR160	5'	-61.5	130	21	57.69231	0.82

miR167	UGAAGCUGCCAGCAUGAUCUA	HRFOW6E01B8W5L	aqc-miR167	5'	-46.7	104	21	51.92308	0.864815
miR169	UAGCCAAGGAUGACUUGCCUG	HRFOW6E02HHZHF	hvu-miR169	5'	-69.9	152	21	45.39474	1.013043
miR1847	UGGCCCACAUGUAAGUGCCGCAAC	HRFOW6E02F3PCZ	osa-miR1847.2	3'	-61.8	96	24	54.16667	1.188462
miR2118	GGCAUGGGAACAUGGAGGAAGG	HRFOW6E01EDC6I	sbi-miR2118-5p	3'	-62.8	218	22	39.90826	0.721839
miR2275	UUUGGUUUCCUCCAAUAUCUCA	HJKAX1S02F9QUH	osa-miR2275a	3'	-38.4	104	22	40.38462	0.914286
miR398	UGUGUUCUCAGGUCACCCCU	HJKAX1S02IGMAR	ahy-miR398	3'	-55.7	119	20	53.78151	0.870313
miR437	AAACUUAAAGAAGUUUGACUU	HRFOW6E02J272H	bdi-miR437	3'	-55.4	211	21	26.06635	1.007273
miR5021	GAAGAAGAAGAAGAAGAAAA	HRFOW6E02GY5DN	ath-miR5021	5'	-60.7	153	20	35.29412	1.124074
miR5049	AGUAUUUUGGUACAGAGGGAG	HJKAX1S02H4BRH	hvu-miR5049b	3'	-61.6	108	21	43.51852	1.310638
miR5067	UCAGCGACAACUAAUAUGGAU	HRFOW6E02HL5QH	bdi-miR5067	3'	-41.5	99	21	39.39394	1.064103
miR5068	AUCGGGUAUAGUGGGUACGGGUAU	HRFOW6E02H92KA	bdi-miR5068	5'	-39.9	103	24	37.86408	1.023077
miR5070	AACUAAGUAGGGUCAGAAGGU	HRFOW6E02JW399	bdi-miR5070	3'	-56.9	142	21	35.21127	1.138
miR5085	AAGGACAUUUUUUGUGGCAUG	HJKAX1S02GQ2PM	tae-miR5085	5'	-50.4	151	21	29.80132	1.12
miR5086	AUAUUGGUGGAAGGCGUGGUA	HRFOW6E01B5HVW	tae-miR5086	5'	-53.2	135	21	34.07407	1.156522
miR5161	UCUGGAUCAGAGGGAGUAAU	HRFOW6E02GP401	osa-miR5161	3'	-46.5	135	20	37.03704	0.93
miR5169	UUUGACCAAGUUUGUAGAAAA	HRFOW6E02H2IV3	bdi-miR5169	5'	-73.7	180	21	26.66667	1.535417
miR5175	AAGUAUUUAGGAACGGAGGGA	HRFOW6E02I3KW6	bdi-miR5175a	3'	-48.8	155	21	36.77419	0.85614
miR5180	UAAGUGUCUCAGUUUUGAACU	HRFOW6E01CPK9C	bdi-miR5180a	5'	-43.6	100	21	34	1.282353
miR5181	UGAUCCAUAAUAAGUGUCGCU	HJKAX1S02IAE2V	bdi-miR5181a	5'	-67.3	122	21	35.2459	1.565116
miR5203	ACUUAUUAUGGAUCGGAGGGA	HRFOW6E02IOMJ2	bdi-miR5203	3'	-71.6	129	21	38.75969	1.432
miR5205	CAUACAUUUUGGGAUGGAGGGAG	HRFOW6E02H1EYQ	mtr-miR5205a	3'	-50.8	117	23	33.33333	1.302564
miR5281	UCUUAUAAUUAGGAACGGAGGGAG	HRFOW6E01CWSVA	mtr-miR5281b	3'	-85	192	24	33.85417	1.307692
miR5387	UGGCACGAACCGGUGCUAAAGGAUC	HRFOW6E01ELY06	sbi-miR5387a	3'	-74.4	156	25	52.5641	0.907317
miR5568	AUCUUAUAAUUUGGAAUGGAG	HRFOW6E02G2G72	sbi-miR5568f-3p	3'	-52.3	177	21	33.89831	0.871667
miR6191	UAGAUUUGUCUAGAUAUGGA	HRFOW6E02IFVP1	hvu-miR6191	5'	-61.3	113	20	31.85841	1.702778
miR6197	UCUGUUCCUAAAUGUAAGACG	HJKAX1S02GC931	hvu-miR6197	5'	-36.1	98	21	31.63265	1.164516
miR6219	CAACCGGGACUAAAGGUGGGUCAU	HRFOW6E01D9CO5	sbi-miR6219-5p	3'	-127.6	170	24	59.41176	1.263366

miR6220	AUGUCUUAUAAUUUGGGAUAGAGG	HJKAX1S02IIDAG	sbi-miR6220-3p	3'	-34.1	98	24	34.69388	1.002941
miR818	ACACCCUUAUAUUAUGGGACGG	HRFOW6E01E40EP	osa-miR818a	3'	-63	121	22	36.36364	1.431818

Accession: 454 sequencing read ID used for microRNA prediction (EBI Sequence Read Archive, accession number ERP002330, http://www.ebi.ac.uk/ena/data/view/ERP002330), Homologous microRNA: miRBase ID of the query microRNA used for microRNA prediction (version 19, August 2012, http://www.mirbase.org/) Location: location of the mature microRNA on the preliminary microRNA hairpin, ΔG: minimum folding free energy of the microRNA hairpin, LP: length of preliminary microRNA, LM: length of mature microRNA, G+C: guanine+cytosine percent of preliminary microRNA sequence, MFEI: minimum folding free energy index, tae: *Triticum aestivum*, bdi: *Brachypodium distachyon*, hvu: *Hordeum vulgare*, osa: *Oryza sativa*, csi: *Citrus sinensis*, aqc: *Aquilegia caerulea*, sbi: *Sorghum bicolor*, ahy: *Arachis hypogaea*, ath: *Arabidopsis thaliana*, mtr: *Medicago truncatula*

APPENDIX N

Characteristics of Selected Preliminary microRNAs Predicted from the Short Arm of Bread Wheat Chromosome 5D

New bread wheat microRNA	Bread wheat mature microRNA sequence	Accession	Homologous miRNA	Location	ΔG (kcal/mol)	LP	LM	GC (%)	MFEI
miR1117	UAGUACCGGUUCGUGGCACGAACC	HJKAX1S01AGLAK	tae-miR1117	3'	-71.5	135	24	54.07407	0.979452
miR1118	CACUAUAUUUUGAAAUGGAGGGA	HJKAX1S01A9CAH	tae-miR1118	3'	-114.8	273	23	36.26374	1.159596
miR1120	ACAUUCUUAUAUUAUGGGACGGAG	HJKAX1S01EOW2N	hvu-miR1120	3'	-56.8	111	24	37.83784	1.352381
miR1121	AGUAGUGAUCUAAACGCUUUUA	HJKAX1S01BYG30	tae-miR1121	3'	-35	97	22	27.83505	1.296296
miR1122	UAGAUACAUCCGUAUUUAGA	HJKAX1S01D7NDW	bdi-miR1122	3'	-23.4	74	20	33.78378	0.936
miR1125	AACCAACAAGACCGACUGCGGCGG	HJKAX1S01ED9TT	tae-miR1125	5'	-79.4	144	24	38.19444	1.443636
miR1127	AACUACUCCCUCCGUCCCAUA	HJKAX1S01A5Q15	bdi-miR1127	5'	-57.4	131	21	33.58779	1.304545
miR1128	AACUACUCCCUCCGUCCCAAA	HJKAX1S01AI0RH	ssp-miR1128	5'	-60.8	144	21	34.72222	1.216
miR1130	UCUGUAACUUAAUAUAAGACG	HJKAX1S01A34BS	hvu-miR1130	5'	-44.8	113	21	34.51327	1.148718
miR1131	UAGUACCGGUUCGUGGCUAACC	HJKAX1S01DQ274	tae-miR1131	5'	-49.4	97	22	54.63918	0.932075
miR1133	AUUAUACUCCCUCCGUCCGAAA	HJKAX1S01CYWO6	tae-miR1133	5'	-67	143	22	38.46154	1.218182
miR1135	UUGCGACAAGUAAUUCCGGACGGA	HJKAX1S01DBQQA	bdi-miR1135	3'	-67.2	140	24	38.57143	1.244444
miR1136	UUGUCGCAGGUAUGGAUGUAUCUA	HJKAX1S01C19Z9	tae-miR1136	5'	-43.8	92	24	35.86957	1.327273
miR1137	UAGUACAAAGUUGAGUCAUC	HJKAX1S01AJCAZ	tae-miR1137	3'	-62.3	82	20	36.58537	2.076667
miR1139	UAGUAACAUAGACUAGUAACA	HJKAX1S01CYHF3	bdi-miR1139	5'	-25.3	87	21	42.52874	0.683784
miR1436	ACAUUAUGGGACGGAGGGAGU	HJKAX1S01AY96G	hvu-miR1436	3'	-51.7	121	21	33.8843	1.260976
miR1439	UUUUGGAACGGAGGGAGUAUC	HJKAX1S01AYKWP	osa-miR1439	3'	-67.1	128	21	44.53125	1.177193
miR167	UGAAGCUGCCAGCAUGAUCUG	HJKAX1S01AGHMM	ahy-miR167-5p	5'	-58.8	113	21	50.44248	1.031579
miR1847	UGGCCCACAUGUCAGUGACACAAC	HJKAX1S01CJWBA	osa-miR1847,2	3'	-58	96	24	50	1.208333

miR2118	GGCAUGGGAACAUGGAGGAAGG	HJKAX1S01B3R27	sbi-miR2118-5p	5'	-65.4	136	22	45.58824	1.054839
miR3700	GACGACCAAAACAGAAGGUCA	HJKAX1S01DS87J	pab-miR3700	5'	-35.5	89	21	51.68539	0.771739
miR395	CUGAAGUGUUUGGGGGAACUC	HJKAX1S01A2W1J	aly-miR395b-3p	3'	-48.6	104	21	50.96154	0.916981
miR482	UUUUUCCAAUGCCUCCCAUUCC	HJKAX1S01AE5TY	gra-miR482	3'	-56.8	133	22	41.35338	1.032727
miR5021	GAAGAAGAAGAAGAAGAAAA	HJKAX1S01D1LIE	ath-miR5021	5'	-31.7	104	20	28.84615	1.056667
miR5049	AAUAUUUCGGUACAGAGGGAG	HJKAX1S01CVFM6	hvu-miR5049b	3'	-64.2	123	21	42.27642	1.234615
miR5067	UCAGCGACAACUAAUAUGGAA	HJKAX1S01ATV4H	bdi-miR5067	3'	-71.7	122	21	40.98361	1.434
miR5068	AUUGGGUAGAGCGGGUACGGGUAU	HJKAX1S01AKTZK	bdi-miR5068	5'	-78.6	93	24	50.53763	1.67234
miR5070	AACUAAGUAGGGUCGGAGGGU	HJKAX1S01C8C0O	bdi-miR5070	3'	-68.6	123	21	39.8374	1.4
miR5175	AAGAAUUUUGGGACGGAGGGA	HJKAX1S01AMO0V	bdi-miR5175a	3'	-93	159	21	35.84906	1.631579
miR5180	AAAGUGUCUCAGUUUUGAACU	HJKAX1S01BS4ER	bdi-miR5180a	5'	-60.6	102	21	40.19608	1.478049
miR5181	UGAUCCAUAAUGAGUGUCACA	HJKAX1S01EK0AJ	bdi-miR5181a	5'	-41	110	21	35.45455	1.051282
miR5203	ACUUAUUUUGGAACGGAGGGA	HJKAX1S01EBP59	bdi-miR5203	3'	-49.1	137	21	32.84672	1.091111
miR5205	CUUACAAUAUGGGACGGAGGGAG	HJKAX1S01AX4HJ	mtr-miR5205a	3'	-51.6	119	23	42.85714	1.011765
miR5387	CGUGGCACGAACCGGUGCUAAAGG	HJKAX1S01DZTO2	sbi-miR5387b	3'	-74.2	158	24	53.16456	0.883333
miR5568	ACUUAUAUUUUGGAACGGAGG	HJKAX1S01A4MRK	sbi-miR5568c-3p	3'	-80	191	21	35.60209	1.176471

Accession: 454 sequencing read ID used for microRNA prediction (EBI Sequence Read Archive, accession number ERP002330, http://www.ebi.ac.uk/ena/data/view/ERP002330), Homologous microRNA: miRBase ID of the query microRNA used for microRNA prediction (version 19, August 2012, http://www.mirbase.org/) Location: location of the mature microRNA on the preliminary microRNA hairpin, ΔG: minimum folding free energy of the microRNA hairpin, LP: length of preliminary microRNA, LM: length of mature microRNA, G+C: guanine+cytosine percent of preliminary microRNA sequence, MFEI: minimum folding free energy index, tae: *Triticum aestivum*, hvu: *Hordeum vulgare*, bdi: *Brachypodium distachyon*, ssp: *Saccharum* sp, osa: *Oryza sativa*, ahy: *Arachis hypogaea*, sbi: *Sorghum bicolor*, pab: *Picea abies*, aly: Arabidopsis lyrata gra: *Gossypium raimondii*, ath: *Arabidopsis thaliana*, mtr: *Medicago truncatula*

APPENDIX O

Selected Preliminary microRNAs Predicted from the Long Arm of Bread Wheat Chromosome 5D

microRNA	Sequence
miR1117	GCCGGUUCGUGUUGAACCUUUAGUACCGGUUCGUG GCACGAACCGGUACUAAAGGGGUGGUGGCAGGCUG GCGUCAGGCCAGGGCCCCACGAUCACCUUUAGUAC CGGUUCGUGGCACGAACCGGUACUAAAGGUCUAAA CUUUAGUACCGG
miR1118	AGUCUAUGUUACUAGUACUCCCUCGUUCCAUAAUG UAGUGCCUAUAAUUUUUGCAAAAAGUCAAACAUUA CAAACUUUGACCGUAUUUAUAGAGAAAAGUAGGUA CAUCUAGAAUACCAAAUGCACACCAUUGGAUACAU CAUGAGUUAUAUUUCAUAAUGUACAUGUUUGGUAU UGUAGAUGUAGACUGUUUCUUCUAUACACUUGGUC AAAGUUGAUAAAGUUUGAUUUUCACAAAAAUCUAU AGGCACUACAUUAUGGAAUGGAGGGAGUACCUUCA UAGUGUGUAGU
miR1120	UACAAAAUGAACCAAUCCCUCUUCCCAUAAUACAA GAGUGUUUUGAACACUGGUGUACUGUACAAAACGU UCUUAUAUUAUGGGACGGAGGGAGCAGCUGUUAAU AUAAG
miR1121	GUAAGAAAUAUAAGAGCGUUUAGAUCACUAGUAUA AGAGCGUUUAGAUCACUACUAUAUUGAUCUAAACG CUCUUAUUCUUUAUGGAGGGAGUAUU
miR1122	AUUCUUGUCUUACAUUUGUUUAGAUACGGAUGUAU CUAACACUAAAAUGUAACUAGAUACAUCCGUAUUU AGACAAAUUUAAGACAAGAAUUU
miR1123	AAGGUGCGCGGAAAAAAUUAUAUGAGACCCAGGUC UUAUAUUUAGCAGGUGAGACCGCCCUGAUGGAUGA CACGUGGCAUUCACAAAUUACAAAGUAUCUAAUCU CUCCCCUCUCCCCCCUCUCCCCCCCCUGAUUUCAAGUG GGGGUGGGUAGAUGCUUUGUGAUUUGUGGAUGCCA CGUGUCAUCCAUCAGGGCGGGUCUCACUGCUAACA CGUGAGACCUGGUCUCAUAGAAUUCUUUUUCAAGG UGCGCC
miR1125	CCAACUUUGACCGUAAAUUUAACCAACGAGACCGA CUGCGGCGGGAGAAAAAAUUAUAUAAUUGAAAAGU UUUUUUGAAUACGAAUUCACUGGUAUAAUUUUUGC UCCCGCUGCAGUUGGUCUCGUUAGUUAAAUUUGUG GUCAAAGUUGAAG
miR1127	ACACUUUUAUUUAUUGUCUGAACUACUCCCUCCGU

	CCCAUAAUGUAAGACGUUUUUCACACUACACUAGU GUCAAAAAGCAUCUUAUAUUAUGGGACGGAGGGAG UAGUAGACAAAGUAUGGAAGGCUGG
miR1128	AAAUGUCUCGAGCUGCUUGAUACUACUCCCUCCGU CCGAAAAUACUUGUCGGAGGAACGAAUGCAUCUAG ACGUAUUUUAGUUCUAGAUACAUCAAUUUUUAUGC AUUUCUCCGACAAGUAUUUCUGGACGGAGGGAGUA GAUUUGUAGUGUCUGGAGUUUUCA
miR1130	UCGAUUUCAAUCUGGUACUCCCUCCGUCUCAUAAU GUAAGACGUUUUUUUACACUACACUAGUGUCAAGA AGCGUCUUAUAUUAUGGGACGGAGGGAGUAGCUUU UAGUCCGUCGUG
miR1131	GCUAGCCGGGCCGCUCUCAUUAGUACCGGUUCGUG GCUAACCUUUAGCACCGGUUCGUGCCACGAACCGG UACUAAUGAGAGUGGUGGCAGGAUGUU
miR1133	AGUCCAGUAGUCUGAAUUGGGUUAUACUCCCUCCG UCCGAAAAUACUUGUCCUAGAAGUGGCUGUAUCUA GAUUUAUUUAGUUAUAGAUACAUUCAUUUUAUUCA UUUGUCUAUUUCCGGACGGAGGAAGUACAUGAUAC CAGACUCUUGACUGAU
miR1135	UGCUAGAAUGCUACUCCCUCCGUUCGGAAUUACUU GUCUUGGAAAUGGAUGUAUCUAAAACUAAAAUACA UCUAGAUACAUCCAUUUCUGCGACAAGUAAUUCCG AACGGAGGGAGUAGGUGGUACUCCAG
miR1136	CUCCCUCCGUUCCAAAUUACUUGUCGCAGGUAUGG AUGUAUCUAGAUGUAUUUUAGUUCUAGAUACAUCC AUUUAUGCGACGAGUAAUUUGGAAACGGUAGGGAG
miR1137	UCCCUCCGUUCCAAAAUAGAUGACCCAACUUUGUA CUAAUUUUGUACUAAAGUUAGUACAAAGUUGAGUC AUCUAUUUUGGAACAGAGGGAGU
miR1139	AGGCUAGUCAUAGUGGAGAGUAACUUACACUAGUA ACAUGCAUAUGUUACUAGUCUAUGUUACUACCUUC AUAGUGGGUAGUGUCAUAUA
miR1436	UUAGUAUCAUUGUAAACUACUUCCUCCGUUUCAUA AUGUAAGACGCUUUUUGACACUAUACUAGUAUGAA AAAAAAGUCUUACAUUAUGGGACGGAGGGAGUAAA UGAUACGACUGUGAAAA
miR1439	UAGGGCGUUGCGAGAUUUGAUACUCCCUCCGUUUC AAAAUAGAUGACUCAACUUUGUACUAUAAAAUUAG UACAAAGUUGAGAAAUCUAUUUUGGAACGGAGUGA GUAUUCGUAUGGUUCUUCAGUCUCC
miR160	AGGUGAAAACAAUGGGAUAUGCCUGGCUCCCUGUA UGCCACUCGCGUAGCUGCCAACUCUCCAAACCCAGC GUUGGCUCUACCGCGGAUGGCGUGCGAGGAGCCAA GCAUGACCGUCUCUCUCUCUCUCU
miR167	AAGAGAAAGCGUGAAGCUGCCAGCAUGAUCUAACU CUUGAACCAGCUAGCUUCCGCGGGUGGUUAGAUCG UGCUGUGACAGUUUCACCCUUUCCCUGUUGGGCA

miR169	GUGGUAAGGGCUUCUUCUGGUAGCCAAGGAUGACU UGCCUGUGGAUAUAUAGCUUGAUCGAUUACACUUA CAUCUCUGCAUGUAUAUAUGUGAUUUCUCACUAUG UACAACAGGCAGUCUCCUUGGCUAGCCCGAGUGGC CCUUAUCUUCCA
miR1847	UACUUAACUGCAGGUGCAGUUGGGUCACUGGCAUG UGGGCCCAAGAGGAAUCUGGCCCACAUGUAAGUGC CGCAACUGCACCUGCUGUUAUGUCAC
miR2118	GCACCAAGAUUAUUUCGUGAUUUCCCGAUGCCUCC CAUGCCUAUUGUUCUUAUGUAUUCCUUCCUUUUUC UAUUUUCAUGCGCAACUAUCAAAACAAAUGUGCAC AUUUAGGUUUAGAUGAUCGGGUUGUGAAGGCAAGU UAGGAAGAGGAAGAAAAUGAACAUCUAGGACCAUU AGGCAUGGGAACAUGGAGGAAGGCCUAAACUAAUU AUUCCAAC
miR2275	AACCUUGCAAGCUGAAUGUGAGAUUUGGAUGGAAC CAAAUCUUCAGCUAUAAUGGCACAAGAUUUGGUUU CCUCCAAUAUCUCAUCUUCAACUGUCCCUGAUGA
miR398	AAGAGAAGGCGAUCCAGAGGAGUGCCACUGAGAAC ACAAGCGCGGGUUCCUUUGUCAGGUGCUCUGGAUC GCAUGCGAUUGUGUUCUCAGGUCACCCCUUUGGGU UGCUUCCCUUGAUU
miR437	GGGGUAUUAUUUGAUGGAAAGUCAAAUUUCUUAAC UUUGAACAAGUUUAGAGCCAAAAAUAUCGACAUCC GCAAUAUUAAAGAAAAAUAUAUGAAAAUUCAUUUC UUGAUGAAUCUAAUAACACCGAUUUGAUAUUAUGA AUUUUGAUUUAUUUCUCUACAAUUUUGAUCAAACU UAAAGAAGUUUGACUUUUCAAACUAAUAAUACGCC A
miR5021	AGGAAGAAAAGAGGAAGAAGGAAGAAGAAGAAGA AGAAAAGGAAGGAGUGGAAAAAGGAAAAUAAGAA GAGGAAGACUUCUUUUUUUUCUUCGAUCUUCUCCU CUAUUCCUUUCUUCUUCUCCUCUAUUUUCUUCUUC GAUCUUUUUUCCUCU
miR5049	ACCACGUGACUAGUACUACUCCCUCCGUACUGAAA UACUUGUAGUUGGGGGCUGUCCCCCAACUACAAGU AUUUUGGUACAGAGGGAGUACUAGAUAAAAAUACU UGA
miR5067	GUUUACCACCCCCCUCUGAUCCAUAUUAAUUGUCG CUGAUUUUAGUAAUUGUACUAAAUCAGCGACAACU AAUAUGGAUCGGGAGUACAACAAUUUCGG
miR5068	AAUAGGAAAAUUUGAAACCCAUCGGGUAUAGUGGG UACGGGUAUGGUUUAGUAUAACCCAUACCCGUGUA CCCAUGUACCUGCAUGUAAUAUGAUUUUUUUAA
miR5070	UCGAAGAACUAAUUUAGUACUUUCCUACCCUUCUU AGUUCAGCCAAAUGAACUAAUUUUUCAAAAGUCAG GUGAUUUCUGAAAUUUUAGUUCAUUUAGUUGAACU AAGUAGGGUCAGAAGGUGUGUACCCCUGAUGGUUU CA

miR5085	ACAAAAAUUUCGGUGAAAAAAAGGACAUUUUUUGU GGCAUGUAUAAAAAAAGAUAAAAAAAAUGCCUUGU GAAUAGAUGUAAUCAAGCAUCGAAAAUUGUCUUUU UUACACAAGCUACAAAAAAUGUCCCUUUUCAUCGA AACUUGGUGCA
miR5086	AUCACGAAAUCAUAUUGGUGGAAGGCGUGGUAAAA AAUAAUGAUGCUUUAAAAUGUUACUUCCAAAAGUA UGUUGGAGCUCUGAUUUUUUUUUGCCACGAGUUGU ACCAAUGUGAUUUCGUGAUGAAAUUUUGCA
miR5161	GCUACUUAUUCAUAAUUUCAUACUCCCUCCGAUCC GUAAUAAGUGUCGCGGGUUUGAAAUAAGUUGAAGU AACCUUAGUUCAAAACUACGAUGCUUAUUCUGGAU CAGAGGGAGUAAUAUAUUUUCAGCUGCAU
miR5169	AGAAGUCAAACUUUGCAAACUUUGACCAAGUUUGU AGAAAAAAAUAUUUAUAUCUACAAUAGCAAAAAUA UAUAAUGCAAAACUACAUCAUAUGAUGAAUCUAGU GAUAUAUGUUUCAAACUUUAGAUGUAAAUAUUUUC UCCACAAACUUGGUCAAAGUUUGUGAGGUUUGACU UUUCA
miR5175	AGUAGCCUUAAUAUUUACUCCCGUCGUUCCUAAAU AUUUAAGGUAUAGAUUCACUCAUUUUGCUCCGUAU GUACUCAUUGCGGUGUAGUCACAAGUUGAAAUCUC UAGAAAGACAAGUAUUUAGGAACGGAGGGAGUAUU UGCUAACUAAUACCA
miR5180	UUAGUCCGUUCGAUCCAUAAUAAGUGUCUCAGUUU UGAACUAAGGUUGAAUUAACCUUAGUUCAAAAUUG CACACAUAUUAUGUAUCAGAGGGAGUACAA
miR5181	AACAUAGUAAGUACUCCCUCUGAUCCAUAAUAAGU GUCGCUCAUUUUGUACUAAGCUAGUACAAACUUUG UACUAAAUGAGCGACACUUAUUAUGGAUCGGAAGG AGUACAAAAUAUGAUGA
miR5203	ACAUGGUUUAUACACUACUCCCUCCGAUCCAAAAU AAAUGUCGCAGUUUUGAACUAAGUUUAGUUCAACC UUAGUUCAAAACUGCGACACUUAUUAUGGAUCGGA GGGAGUAGAUCAGAUCGAAGAGAA
miR5205	AAUUAAUGAUAAUAUAGACUCCCUCCGCCCCAAAA UGUAAGACAUUUUUAACACUAUAUAGUGUCAAAAA ACGUCAUACAUUUUGGGAUGGAGGGAGUAUAUGUU AACUAAUCCUCA
miR5281	AUAAUAGCUAAAUUCUUACUCCCUCUAUUCAUAAA UAUAAGACCUUUUAGAGAUUCCACUAUAGACUACA UAGGGAGCAAAAUGAGUGAAUCUACACUCUAAAAC AUGUCUAUAUCCGUAUGUAGUCCAUGGUAGAAUCU CUAAAAGGUCUUAUAAUUAGGAACGGAGGGAGUAC AUAUCAGAUAAGCAUUG
miR5387	UUUAUAUAGGACGACCUUCAGUACCAGUUCGUGGC ACGAACCGGUACUAAAGGUGCUGGAGGGGCCCCAC UCUGACAACAUCCUGCCACCACUCUCAUUAGUACC GGUUUGUGGCACGAACCGGUGCUAAAGGAUCGCCA UGAACCGGUACUAAUG

miR5568	GGCUCAAAUUAGUACUACCUUCGUUCCAAAUUAUA AGAUGUUUUGGAUAUUUCAAUAUGGUCUACAUAGA CUGAAAUGAGUGAACAAACACACUAAAACACGUCU AUAUACAUCCGAUUCUCAUUUUGUUUACAAGAUCU UAUAAUUUGGAAUGGAGGGAGUACGUUCUGGUUGU AG
miR6191	CCGUCCCAAAAUUCUUGUCUUAGAUUUGUCUAGAU AUGGAUGAAUCUAAUACUAAAACAUGACUUGAUAC AUCCGUAUUUAGACAAAUUUAAGACAAUAAUUUUG GGAUGGAG
miR6197	UUACUAGUAAAGGUACUGAUUCUGUUCCUAAAUGU AAGACGUUUUUAGAAGUGCAAAAACGUCUUACAUU UAUGAACAGAGGGUAGAUUAUACGAUUU
miR6219	GGCACAAACCGGGACCAAGUGCACCCGUUUAGUCC CGGUUAGGUGCCACCAACCGGAACCAAAGGUCAAC UUUUUCGGCAGCCCAAUGGGUGGGAAGCAGAGGCC UUUGGUCCCGGUUGGUGGCACCAACCGGGACUAAA GGUGGGUCAUUUGGUCCCGGUUGGUGCCAC
miR6220	AAAUACUACCUCCAUCUCAAAUCAUAAGACAUUUU GGGUGGGCUAUUACCAAAAAUGUCUUAUAAUUUGG GAUAGAGGAAAUAGAUGAGUUGGAAUGG
miR818	GUUUUUUUACUACUCCCUCCGUCCCAUAAUAUAAG AGUGUUUUUGACACUUGUAGUGUCAAAAACGUUAA GUGUCAAACACACCCUUAUAUUAUGGGACGGAGGA AGUAGAAAGUAGUAUA

APPENDIX P

Selected Preliminary microRNAs Predicted from the Short Arm of Bread Wheat Chromosome 5D

microRNA	Sequence
miR1117	AGGACGACCUUUAGUACCGGUUCGUGAUACGACCCGGUACUA AAGAUGCUGGAGGGGCCCUGGACUAACAACAUCCUGCCACCA CUCACUUUAGUACCGGUUCGUGGCACGAACCGGUGCUAAAAG GUUCACCAC
miR1118	UGUGUAUAUAUAUAGUACUCCCUCUAUUUUAUAACGUAGUGC UUCCUCUAUCCCCGUGCUUUAAUUUUGACUAUGAAUUUAACU ACCAAGAUCGAUUACGGCGGGAACAAAAAUUAUAUUAGUGAA UUCGUAUUCGAAAGAAGUUUUCAAUUAUAUAAUUUUUUCUCC CGUCGCAGUUGGUCUCGUUGAUUAAAUUUAUGGUCAAAGUUG GACCUCGAAAAGUGCGGGCGCACUAUAUUUUGAAAUGGAGGG AGUACUACAAGCAGACACGUA
miR1120	UCUCCGUCCCAAAAUUCUCUCCGUCCCAAAAUAUAAGAACGU UUUUGAUACUAGUGUAGUGUCGAAAACAUUCUUAUAUUAUGG GACGGAGGGAGUAGAUGUUAGAGAUUU
miR1121	ACUAACUAAUACUUAAUAUAAGAGUGUUUAGAUCACUAAAGU UAAUAUAAGAGCGAGUAGUGAUCUAAACGCUUUUAUAUUAGU UUACAGAAGGAGU
miR1122	UUCUUGUCUUAGAUUUUGUCUAGACACGGUUGUAUCUAACAC UAAAACGUACUAGAUACAUCCGUAUUUAGAC
miR1125	ACCAUAAAUUUAACCAACAAGACCGACUGCGGCGGGAGCAAA AAUUAUAUAAUUGAAAACUUCGUUUGAAUACGAAUUCGUUGG UAUAACUUUUGCUCCCGCCACAGUCGGUCUCAUUGGUUAAAU UUAUGGUCAAAGUUGAAG
miR1127	AAGGACAAACUUUGUUUAUGAACUACUCCCUCCGUCCCAUAA UAUAAGAACGUUUUUUACACUACACUAAUGUCAAAAACGUUC UUAUAUUAUGGGACGGAGGAGAGUACAUAAUAUACUUUAAAC AUGCA
miR1128	GAAAUAGUGAUACAUGUAAUAACUACUCCCUCCGUCCCAAAA UAAGUGUCUCAACUUUGUACUAACUUUAGUACAAAUUUGUAC UAAGGUUGAGACAGAUAUUUUGGGACAGAGGGAGUAUAUGU UAGUUUAGUCAUGUGACAU
miR1130	GCAGACAAUUUAAUACUCCCUCUGUAACUUAAUAUAAGACGU UUUUUCACACUAUGACAUUGUCAAAAAAUGUCUUCUCUUAAG UUACAGAGGGAGUAGCACAACAGAAUCUG
miR1131	GGCUAGCCGGGCCUCUUCAUUAGUACCGGUUCGUGGCUAACC UUUAGCACCGGUUCGUGCCACGAAUCGGUACUAAUGAGAGUG GUGGCAGGAUGUU

miR1133	UUUACGGAAUACAUGCCUACAUUAUACUCCCUCCGUCCGAAA UAAGUGACUCAAGUUUGUACUAACUUUGUACUAAACUUAGUA CAAAUUUGAGUCACUUAUUUUGGGACGAAGGGAGUAUUUAUG AACGGGAGCUAGUGCCA
miR1135	UCCCUCAAAUGUACUCCCUCCAUCCGGAAAUACUUGUCAAAG AAAUGGAUGUAUCUAGAUAUAUUUUAGUUCUAGAUACAUCCG CUUUUAUCCAUUUUGCGACAAGUAAUUCCGGACGGAGGGAGU AUAUAUCUAGCAUC
miR1136	GAAUUAUUUGUCGCAGGUAUGGAUGUAUCUAGAUGUAUUUU AGUUCUAGAUACAUCCAUUUCUGCGAUGAGUAAUUUGAAACG GAGGGAGUA
miR1137	UUCCUCCGUUUCAAAAUAGAUGACUCAACUUUGUACUAUAGU UAGUACAAAGUUGAGUCAUCUAUUUUGGAACGGAGGGAGU
miR1139	CAACGGCAAACUUCGCGCAGUAGUAACAUAGACUAGUAACAU AUGCAUGUUACUAGUCCAAGUUACUCACCACUAUGACCAGCC UAA
miR1436	UUAAAAAAUCUAAAUAUUACUCCCUCCGUCCCAAAAUAUAAG ACGUUUUUUGAUUCUAGUGUAUUGUUAAAAAAUGUCUUACAU UAUGGGACGGAGGGAGUAGAAUGGUUCCUGCAAUUGC
miR1439	AGCUCUUUCUCUCUCUCUGCUACUCCCUCCGUUCCAAAAUAG AUGACUCAACUUUAGUAUAAAGUUAGUACAAAGUUGAGUCAU CUAUUUUGGAACGGAGGGAGUAUCUCUCACGGGCCUCCCUCU CC
miR167	CAAGGUGCACCACAAGCUGGUGAAGCUGCCAGCAUGAUCUGA UGACCUAAGUCAUGGAUCAGAAUCCAUGUCAAUCAGGUCAUG CUGGAGUUUCAUCUGCUGGUCGGAGCAC
miR1847	UUACUUAACUGCAGGUGCAUUGGGUCACUGACAUAUGGGCAC AACAGGAGUCUGGCCCACAUGUCAGUGACACAACUGGACCUG CAGUUAAGUCAG
miR2118	AUGAGCAUCUAAGAGCAUUGGGCAUGGGAACAUGGAGGAAGG CCUAAACUAGUGUGUCCCUGUGCAACUAGGGUCGAACAUUUG UUUUGGAAAUUUCCCGAUGCCUCCCAUGCCUAUUGUUCUUAG GUGUUUGAUA
miR3700	GUGGCCUGGGCAGACACCUAGACGACCAAAACAGAAGGUCAU AGGAUCUAACGACCUUUUGUUUGGUCGUUGCCUUCCACGACC UGAGA
miR395	CGAGUUUGGCCUCGUCAGGAGUUCUCUUCAAGCACUUCGGGA CGCUUCUGUUCGAGAACUUCUCUGAAGUGUUUGGGGGAACUC UUGGUGUCACCAAGCAUUCA
miR482	GAGGGUCCAAGAGUAGUAUGAAUGGGAACAUUGAGGAAAACA AGAGCUUUCUAGAUUUCAUCUCCUAAGGAAUUAGAGAUCUAG UUCUGUGUUUUUCCAAUGCCUCCCAUUCCUAUUGUUCUUGGG UGCUCCU
miR5021	UAGAAGAAGAAGACUAGCUAGAAGAAGAAGAAGAAGAAAAA GAAGAAAAAAAAUUUCUUCUCUUUCUUUUUUCUCCUCUCCUC UUCUUUAUCUUAAUUCUUCUU

miR5049	AUAGGGGCAAUUGUACUACUCACUCUGUACCGAAAUAUUUGU AGCUGAGGGAAUGUUCUCCAGCUAUGUUCCCCCAGCUACAAA UAUUUCGGUACAGAGGGAGUACUACGCAAUUUACUUCAU
miR5067	AAAUAAGUACUCCCUCCGUUCCAUUUUAGCUGUCGCUCAAAC GGAUGUAUCUAGCACUAAAUACGUCUACAUACAUCCGUUUCA GCGACAACUAAUAUGGAACGGAGGGAGUAACUGUUAAU
miR5068	AUAGGGAAAAUCUCAAACCCAUUGGGUAGAGCGGGUACGGGU AUGGGUAUGUGAUCCCAUACCCGUACCCGCUCUACCCAAUGG GUUUGAGA
miR5070	AUUGGGAACCUAAAGGGUACCUUCUGACCCUUCUUAGUUCAA CCAAAUAAACUAAUUUUUCCUAAUUUAGUUCAUUCAGUUGAA CUAAGUAGGGUCGGAGGGUACCCUGGAGGUGCCAAAUUU
miR5175	UUCACCGAUAUCUUAUACUCCCUCCAUCCCAAAAUUCUUGUC UUAGAUUAGUCUAGAUACGGAUGUAUCUAAUACCAAAACGUG ACUUGAUACAUCCGUAUUUAGACAAAUCUAAGACAAGAAUUU UGGGACGGAGGGAGUAUUUCAUUGGAUUUAACA
miR5180	CUACUCCCUCUGAUCCAUAAAAAGUGUCUCAGUUUUGAACUA AGGUUGAACUAGCCAUAGUGCAAAACUGUGACACUUAUUUUC GGAUCAGAGGGAGUAGUU
miR5181	GCUUACUGUAGUACUAUCUCUGAUCCAUAAUGAGUGUCACAG UUUUAAACUAACCCCAGUUCAAAACCGUGACACUUAUUUUGG AUAAGAGGGAAUAAAUUAUUUUAGGG
miR5203	AUCAUUUUUUUUCUUAGCUGCCUCUGUUUCCAAAAUAAGUGU CAACUUUAGUAGCAGUAGUACAACUUUGUAUUAAAGUUUUCA CAAAGUUGAGACACUUAUUUUGGAACGGAGGGAGUACAUAUU UGCUAUGAUCU
miR5205	CGUUUUGCCUAAAAAUUACUCCCUCCGUCCCAUAAUGUAAGA CGCUUUUUCACACUAGUGUACUGUCAAAAAACGCCUUACAAU AUGGGACGGAGGGAGUAUCAAGCUAGCUUGUGACA
miR5387	AGGGUUUAUGUAGGACGACCUUUAGUACUGGUUCGUGACACA AACCGGUACUAAAGGUGCUGGAGGGGCCCCAGGACUGACAAC AUCCUGCCACCACUCACUUUAGACCUGGUUCGUGGCACGAAC CGGUGCUAAAGGUCACCACGAACCGGUACUAA
miR5568	CUUCUUUAAUAUGUACUCUCUCCGUUCCUAAAUACAAGUCUU UGUAUAGAUUCCACCAUGGACUACAUAUGGAGCAAAAUGAGU GAAUCUACACUCUAAAAUGUAUCUGGAUACAUCUGUAUGUGA UCCAUAGUGGAAAUCUCUACCAAGACUUAUAUUUUGGAACGG AGGGAGUAUUAUAGUAGUACAGU
miR6191	CUGUCCCACAUUUCUUUUCUUAGAUUUGUCUAGAUAUGGAUA UAUCUAAUACUAAAAUGUGACUUGAUACAGCCGUAUCUAGAC AAAUCUAAGACAAGAAUUUUGGGACAGAG
miR6197	UUCCCUUAGUAAGUACUCCCUCUGUUCCUAAAUGUAAGACGU UUUUGCAGUUCGUUAAAAAACGUCUUACAUUUAGGAACAAAG GGACUAUAUGCUGGCAAGGGG

miR6219	UGGCACCAACAGGUACCAAUGCCCCCUUUAGUCUCGGUUGGU GCCACCAACUGGGACCAAAGGUCUCUUUUUAGCAGCCCAAAG GGCGGGAAGCGGUGGCCUUUGGUCCCAGUUGGUGGCACCAAC CGGUACUAAAGGGGGGACAUUGGUACCGGUUGGUGCCACG
miR6224	AACUCUUUACAUAGUACUCCCCCUGUCCCAUAAUAUAAGAAC AUUUUUCAAACUAUGUUAGCUUGAAAAAUGUUCUUAUAUUAU AGGACGGAGGGAGUAGUAUGUACCGUAAUUU
miR834	GCUACCGCGCUACAGCUAAGUUGUAGCAGUAGCGCUGGUAAG GGCAGCGCUACUGGUAAGUAUGGUUAGCAGUAGCGCUGCCUU GACCAACGCUACUGCUAACUCUCUGUAGCGCUUGAACAAA
miR845	CUAGUCGAUAACUAGCACUUUCAAUUGGUAUCAGAGCAAGGU ACUCCCUUGUUCUGUGUGAUCCAGUUGAAUGAACUUCUUGAC AUCA
miR950	CAGAGAUGGGGACGGUCCUCAAGGAAUCGCCUGGACCCCCAG AGCCUCCCGAGGCUCAGGGCCCCGGUGGUUCAUGAGGAACAA CCCCUUCGCCGCG

APPENDIX R

Predicted Targets of Putative microRNAs of Bread Wheat 5D Chromosome Long Arm

microRNA	Target sequence	Target protein
miR1117	TC413428	Chloroplast methionine sulfoxide reductase B2 precursor
	CK216289	Histone H2A.1
	CK211158	30S ribosomal protein S17, chloroplast precursor
	CK211728	PS II 10 kDa protein
	CK211670	Cytochrome c
	CK211508	Trypsin inhibitor
	TC391048	UDP-glucose dehydrogenase
	TC405271	RNA recognition motif (RRM)-containing protein-like
	TC435744	Ferredoxin-sulfite reductase precursor
	CA723738	Aquaporin
	TC455991	Glucosamine-6-phosphate acetyltransferase
	TC451865	Alcohol dehydrogenase ADH1
	TC408973, TC383766	Beta-carotene hydroxylase
miR1118	CK196934, TC369361	Calmodulin TaCaM2-1
	TC418995	Glutathione S-transferase 2
	TC437336	Ribulose bisphosphate carboxylase large chain
	TC391749	Protein HVA22
	AL821011	Sugar transporter
	TC370181	Calmodulin TaCaM2-3
	TC440827	F-box domain containing protein-like
	TC379298	Geranylgeranyl hydrogenase
	CK216826	Cold-regulated protein BLT14
	TC373115	Leucine zipper protein-like
	TC452368	GTP-binding protein
	TC392645, TC413383, TC418029	Low temperature and salt responsive protein
	TC411582, TC411582	JD1 calcium ion binding, transferase activity, transferring acyl groups
	DR732284	ERN3
miR1120	TC402657	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
	TC390499, TC449066	S-adenosylmethionine decarboxylase proenzyme
	CJ668032	Leucine Rich Repeat family protein
	TC373115	Leucine zipper protein-like

	TC405470	Protein translation factor SUI1 homolog
	CK215752	High light protein
	TC453849	U2AF large subunit
miR1121	TC446821	Preprotein translocase secA subunit
	CJ839181	Ubiquitin carrier protein
	TC372427	Catalase-1
	TC410034	Actin-related protein 2/3 complex subunit 5
miR1122	CD883901	Fusarium resistance protein I2C-5-like
	AL819672	Sodium/hydrogen exchanger
	TC388938	Eukaryotic peptide chain release factor subunit 1-3
	CJ531707	Betanidin 6-O-glucosyltransferase-like protein
miR1123	CD882751, TC455169	Rab3-GAP regulatory domain-like
	TC433565	Polygalacturonase inhibitor
	TC379745	WRKY DNA binding domain containing protein, expressed
miR1125	CD454825	Plasma membrane proton ATPase
	CK151922	Tryptophan synthase, alpha subunit (TSA1) precursor
	TC402418, TC382890	Farnesyl pyrophosphate synthetase (FPP synthetase) (FPS) (Farnesyl diphosphate synthetase)
	GD187740	Gag-pol polyprotein
miR1127	TC439689	Calreticulin-like protein
	TC409696	Branched-chain-amino-acid aminotransferase-like protein 1
	TC375086, TC400854	Universal stress protein family protein, expressed
miR1128	TC439689, BE516586	Calreticulin-like protein
	TC374504	UDP-D-glucose epimerase 1
	TC405058	Protein kinase domain containing protein, expressed
	TC415559	Malic enzyme
	CK198514	Peroxidase 6
	TC387297	Pollen allergen Lol p 2-A
	BQ838905	Early nodulin protein
	GH726155	Type 1 non-specific lipid transfer protein precursor
	DR731606	DEAD-box ATP-dependent RNA helicase 42
	TC409696	Branched-chain-amino-acid aminotransferase-like protein 1
	BE442673	Methylthioribose kinase
	TC448466	Glycine-rich_protein_(Aa1-291) precursor
	TC369045	CBFIVd-B22

	TC425744	Truncated COL1
	TC381299	Gamma-secretase subunit PEN2-like
	TC434949	Sialyltransferase like protein
	TC369447	Zinc finger protein ZFP-like
	CJ868604	Transcriptional activator-like
	CJ907078	Brain protein 44-like
	CK168772	Monodehydroascorbate reductase
miR1130	TC381627	Cinnamyl-alcohol dehydrogenase-like protein
	CD911932	Endonuclease-like
	CJ864198	NB ARC domain containing protein
	TC378043	Seryl-tRNA synthetase family protein, expressed
	TC395633	Ethylene receptor-like protein
	CJ861664	Phosphatidylinositol transfer-like
	TC461034	Ubiquitin conjugating enzyme 7 interacting protein 4-like
	TC440127	RNA polymerase II complex component SRB7 protein-like
	CK211052	NADPH-cytochrome P450 reductase
miR1131	CA707384, CA678412	Chlorophyll a-b binding protein, chloroplast precursor
	CV522963	Obtusifoliol 14alpha-demethylase
	TC446901	Sucrose-phosphate synthase 9
	TC431084	Metallothionein-like protein type 3
	CK212340	Hydrolase, alpha/beta fold family-like
	TC432746	Potassium channel protein
	CA676769, CA679919	Chlorophyll a-b binding protein 1, chloroplast precursor
	TC398242,TC395320	Beta-amylase
	TC372505	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplast precursor
miR1133	TC394164	Phosphatidylinositol transfer protein, expressed
	BE516586	Calmodulin-like protein
	CK198514	Peroxidase 6
	BQ838905	Early nodulin protein
	GH726155	Type 1 non-specific lipid transfer protein precursor
	DR731606	DEAD-box ATP-dependent RNA helicase 42
	TC368568	SET domain protein
	TC439651	Selenium-binding protein-like
	TC454714	ATP sulfurylase
	CJ943226	Hydrolase, alpha/beta fold family protein,

		expressed
	TC434949	Sialyltransferase like protein
	TC391404	Cysteine synthase (O-acetylserine sulfhydrylase) (O- acetylserine (Thiol)-lyase)
	TC395662	Serine/threonine-specific receptor protein kinase-like
	TC374504	UDP-D-glucose epimerase 1
	TC389947	Ribonucleoside-diphosphate reductase
	TC369045	CBFIVd-B22
	CJ562562	Phytase
	TC388253	Speckle-type POZ protein-like
miR1135	CD888735	Delta1-pyrroline-5-carboxylate synthetase
	CD931690	Cytochrome P450
	TC433142	Alliin lyase-like
	TC445105, TC394690, TC404007, TC441900, TC424561, TC401095	Monodehydroascorbate reductase
	TC374375, TC376089	Mitochondrial 2-oxoglutarate/malate translocator
	TC383624	Peroxidase 6
	TC456258	Importin beta-like protein
	CJ560586	Ribosome recycling factor, chloroplast precursor
	TC395537	At1g66240/T6J19_6 (metal ion binding)
	TC374632	Fructan exohydrolase
	TC390088	Coatomer subunit zeta-1
	CJ531707	Betanidin 6-O-glucosyltransferase-like protein
	TC418688	Arogenate dehydratase mutant
miR1136	TC405058	Protein kinase domain containing protein, expressed
	BJ263423	Early light-inducible protein ELIP
	TC374375, TC376089	Mitochondrial 2-oxoglutarate/malate translocator
miR1137	TC418521	Expansin-like A1 precursor
miR1139	TC397585	Transcription factor Myb1
	CJ636238	Glycine cleavage system H protein, mitochondrial precursor
	CK158705	Quinone reductase
	TC436825	Sodium/hydrogen exchanger
	TC436537	General substrate transporter
	TC386006	Cyclin-dependent kinases regulatory subunit 1
	TC391844	Gamma-TIP-like protein
	TC387148	SAR DNA binding protein

	TC405700	C3HC4 zinc finger containing protein
miR1436	TC453849	U2AF large subunit
	TC433753	Proline-rich spliceosome-associated protein-like
	CV762246	Glycosyltransferase
	BE425224	Receptor-like kinase 17 precursor
	CK161922	D-xylose-proton symporter-like 2
	CK196934	Calmodulin TaCaM2-1
	TC370176	Resistance-related receptor-like kinase
	TC418995	Glutathione S-transferase 2
	TC437336	Ribulose bisphosphate carboxylase large chain
	TC373115	Leucine zipper protein-like
	CV779218	RAB1X
miR1439	TC370181	Calmodulin TaCaM2-3
	TC369361	Calmodulin TaCaM2-1
	TC405876	Ring zinc finger protein-like
	TC379298	Geranylgeranyl hydrogenase
	CK207929	U2AF large subunit
	TC452182	Glyoxalase II
	TC456061	Delta 1-pyrroline-5-carboxylate synthetase
	CJ796908	F1E22.16
	TC406001	Histone H2B.1
	TC425204	Phytepsin precursor (Aspartic proteinase)
	CV764254	Glycosyltransferase
	TC383543	Citrate synthase
	TC383916	Glutathione transferase
	TC380076	BRI1-KD interacting protein 109
	TC394286	CTV.22 transcription cofactor activity
miR160	TC412104, TC416747	Auxin response factor 8
	TC425315	Auxin response factor 22
	TC405771	Histone H2B.2
	CJ806355	Tubulin folding cofactor B
	CK213447	Chlorophyll a-b binding protein, chloroplast precursor
miR167	TC407440,TC399806, TC408373, TC375994, TC433708,TC448400	Proteasome subunit alpha type-5
	TC407798	Ribulose bisphosphate carboxylase small chain clone 512
	TC379113	Auxin response factor 9
miR169	TC426343, TC370257	CCAAT-box transcription factor complex WHAP12
	TC430923	CCAAT-box transcription factor complex

		WHAP6
	CA664884	Photosystem II 10 kDa polypeptide, chloroplast precursor
	TC377727	RAPB protein
	TC387436	CCAAT-box transcription factor complex WHAP12
miR1847	TC369329	WRKY transcription factor
	TC416401	Alpha tubulin-2A
miR2118	TC447958	Light stress-responsive one-helix protein-like
	TC439977, TC415030	GTP-binding protein-like
	TC403906	Triticain beta
	TC435268	Kinase R-like protein
	TC394406, TC422650	DNA-binding protein family-like
	CV779069	Light induced protein like
	TC425646, TC421071	NBS-LRR type RGA
	GH725089	S-like RNase
miR2275	TC401833	NPR1-like 1
	CA627100	COP8-like protein
	TC440979, TC385932, TC424445	Nicotianamine aminotransferase A
	TC401011	Cell Division Protein AAA ATPase family
miR398	CA702164, CA709966, CA635382, CA669113, CA665414, CA614433,CA606891, CA700248, CA598436, CA682577, CA598953, CA663864, CA650645, CA707648, CA607185, CA647068	Superoxide dismutase [Cu-Zn]
	CA671304, CA712263, CA602586, CA613247, CA609950, TC419428, CA606248	Superoxide dismutase [Cu-Zn] 4A
	CV770576	Fructose-bisphosphate aldolase
	TC437415	Alpha-glucan phosphorylase, H isozyme
miR437	TC397956	Bifunctional nuclease
	BQ579749	Phosphoglucomutase
	TC386008	Aquaporin
	TC389697	Zinc finger protein-like
	TC421920	Avenin precursor
	BJ323295	Receptor protein kinase
miR5021	CK205372	Expansin EXPA12
	TC373827	Pistil-specific extensin-like protein precursor

	TC424354	FERONIA receptor-like kinase
	TC402816, TC387108, TC438021	GASA2-like protein
	CA613725	Serine/threonine-protein phosphatase PP1
	CK156225	MAR-binding protein
	BJ221255	TO71-3
	CD911426	Membrane related protein
	TC403644	Gt-2
miR5049	TC436590	Lipid transfer protein 4
	CD903703	SGRP-1 protein
	TC433193	1-aminocyclopropane-1-carboxylate oxidase
	GR303850	Carboxylesterase-like
	TC421317	Spermidine synthase 1
	TC410073, AL819672	Sodium/hydrogen exchanger
	TC444618	Abscisic stress ripening-like protein
	TC416512	Citrate synthase
	TC398706	PG3
	TC394286	CTV.22
	TC423054	T6A9.6 protein
	CA741812	Pollen allergen Lol p 2-A
	TC413664, TC406001	Histone H2B.1
	CA711590	Nucellin-like aspartic protease
	TC403828	DNA-binding protein
	CD931690	Cytochrome P450
	CJ531707	Betanidin 6-O-glucosyltransferase-like protein
	TC406957	Homeobox domain containing protein, expressed
	TC394191	Susceptibility antioxidant protein
	TC382066	Protein phosphatase 2C
	TC394281	40S ribosomal protein S15
	TC460644	P-type R2R3 Myb proteind
	TC436262	Cyclic nucleotide gated channel
	TC426391	Protein phosphatase type 2-C
	TC421485	TAP42-like family protein, expressed
	TC431549	Enhancer of rudimentary homolog
	TC377619	Lecithin:cholesterol acyltransferase
	TC384659	Chloroplast 50S ribosomal protein L31
	TC414990	Low molecular mass early light-inducible protein HV90, chloroplast precursor
	TC461985	MAP kinase
	TC421291	CMP/dCMP deaminase, zinc-binding
	TC438644	Wpk4 protein kinase

	TC423859	Short-chain dehydrogenase/reductase protein-like
	TC414519	Acyl carrier protein 3, chloroplast precursor
	TC379110	Calnexin
	BJ257353	Peroxidase
	TC382725	Lon protease homolog
	TC380088	NBS-LRR type resistance protein-like
	CJ530860	SNF7 protein-like
	BJ308107	AGO1-2 nucleic acid binding
	CK207999	Major pollen allergen Hol l 1 precursor
	TC401095, TC424561	Monodehydroascorbate reductase
miR5067	TC438644	Wpk4 protein kinase
	CK155004	Geraniol 10-hydroxylase
	BJ257353	Peroxidase
	TC400158	Sigma-54 dependent response regulator
	TC374008	Calnexin
	BJ307801	Transducin family protein-like
	TC453848	Cytochrome P450 family protein, expressed
	TC422449	Inositol 1,3,4,5,6-pentakisphosphate 2-kinase
miR5068	CV066196	Allergen C-C
miR5070	TC438766	Replication protein-like
	TC411423, TC380718	Initiator binding protein
miR5085	CJ661506	Target of rapamycin
miR5086	CA731664	Extracellular invertase
	DR739186	Sucrose:sucrose 1-fructosytransferase
	CA735478	Type II LHCI
miR5161	CA647968	LEM3-like
	CJ530860	SNF7 protein-like
	CK204669	6-phosphogluconolactonase
	TC387475	Pyruvate kinase
	TC414519	Acyl carrier protein 3, chloroplast precursor
	TC379110	Calnexin
	CV066415	Aspartic proteinase
miR5169	CD452777	F6I1.13 protein
	TC395865	Class I chitinase
	TC444765	U1 snRNP-interacting 70 kDa protein
	TC440827	F-box domain containing protein-like
	TC432055	Cytochrome P450
	TC392729	Pentatricopeptide (PPR) repeat-containing protein-like
	TC395705	Ent-kaurene oxidase 1
miR5175	TC394281	40S ribosomal protein S15
	TC387478	Methylenetetrahydrofolate reductase

	TC431549	Enhancer of rudimentary homolog
	TC377619	Lecithin:cholesterol acyltransferase
	TC370810	RadA-like protein
	TC426391, TC382066	Protein phosphatase type 2-C
	DR731536	DEAD-box ATP-dependent RNA helicase 50
	TC380076	BRI1-KD interacting protein 109
	TC416752, TC438186	Harpin-induced protein 1 containing protein, expressed
	CJ554691	Phosphoenolpyruvate
miR5180	CD903828	Starch branching enzyme I precursor
	TC413536	TIA-1 related protein
	CJ608275	Photosystem II polypeptide
miR5181	CJ596436	Translin family protein, expressed
	BE412130	Ribulose bisphosphate carboxylase small chain
	TC374061, TC384192	Nuclear movement protein-like
	TC411114	Membrane protein COV-like
	TC431308	Dehydrin 11
	CA660270	Beta-glucosidase aggregating factor-like protein
	CA635916	Branched-chain amino acid aminotransferase-like
	BQ744502, DR740657, TC395009	Hin1-like protein
	CA731524	Carbonic anhydrase, chloroplast precursor
miR5203	TC385465	Non-specific lipid-transfer protein
	CJ530860	SNF7 protein-like
miR5205	TC412474	60S ribosomal protein L44
	TC459951	Protein kinase domain containing protein, expressed
	TC452182	Glyoxalase II
	TC433753	Proline-rich spliceosome-associated protein-like
	TC453849	U2AF large subunit
	TC398674	Phytoene dehydrogenase, chloroplast/chromoplast precursor
	TC389043	Malate dehydrogenase [NADP], chloroplast precursor
	AL821953	CHY zinc finger family protein, expressed
	TC383983	Utp14 protein, expressed
	DR732905	MIKC-type MADS-box transcription factor WM22A
	CK214985, CK211600,CK216047, CK214076	High light protein

	CN012529	Mitochondrial transcription termination factor-like protein
	TC420918	Arf6/ArfB-family small GTPase
	CD879785	Probable protein ABIL1
	TC430550	Aldehyde oxidase-2
	TC407053	NADPH-cytochrome P450 reductase
	TC413453	F-box domain containing protein, expressed
	TC402657	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
	CD880846	Flowering promoting factor-like 1
	TC449066,TC390499	S-adenosylmethionine decarboxylase proenzyme
	CD879878	Serine-threonine protein kinase
	TC377933	ATP dependent Clp protease ATP-binding subunit ClpX1
	TC406931	Ribosomal protein L7
	CJ883403	Cysteine synthase
	CJ563368	Lipid transfer protein-like
	TC377545	Ubiquitin-conjugating enzyme-like protein
	TC384047	Glycosyltransferase
miR5281	AL821953	CHY zinc finger family protein, expressed
	TC398674	Phytoene dehydrogenase, chloroplast/chromoplast precursor
	TC389043	Malate dehydrogenase [NADP], chloroplast precursor
	BQ166729	HAT family dimerisation domain containing protein, expressed
	TC383983	Utp14 protein, expressed
	TC415094	Cell division inhibitor-like
	TC424763	Protein kinase domain containing protein, expressed
	TC425550	Lipase class 3-like
	TC430886	SMC5 protein
miR5387	TC437702	Histone H3.2
	BE637541	LEA protein
	TC387640	Heterogeneous nuclear ribonucleoprotein A2/B1-like
miR5568	TC393805	Protein kinase
	TC390967	Glutaredoxin-C1 precursor
	DR732905	MIKC-type MADS-box transcription factor WM22A
	AL821953	CHY zinc finger family protein, expressed
	TC405376	Serine carboxypeptidase family protein, expressed

	CK211600, CK216047, CK214985, CK214076	High light protein
	TC392962	Serine protease-like protein
	TC379065	Proteinase inhibitor
	TC452003	Subtilisin-chymotrypsin inhibitor 2
miR6191	CJ868604	Transcriptional activator-like
	TC371963	Alpha-1,2-fucosidase
	TC453681	Ribosomal protein L15
	TC400735	SET domain containing protein, expressed
miR6197	TC440668	Ribosomal Pr 117
	TC383983	Utp14 protein, expressed
	TC446402	Glutathione gamma-glutamylcysteinyltransferase 1
	TC380096	haloacid dehalogenase-like hydrolase family protein
	AL821953	CHY zinc finger family protein, expressed
	TC391197	Leucine Rich Repeat family protein, expressed
	TC442517	Protein HVA22
	TC438131	Ice recrystallisation inhibition protein
	TC415220	Kinase, CMGC CDKL
	TC379438	Germin-like protein 1 precursor
	TC397912	Adenosine diphosphate glucose pyrophosphatase precursor
	TC381279	Metallo-beta-lactamase-like
	TC452182	Glyoxalase II
miR6219	CO348282	Mono-or diacylglycerol acyltransferase
	CA697004	Auxin-repressed protein-like protein ARP1
	TC376658	Peroxidase 8
	CA617623	Vacuolar ATP synthase 16 kDa proteolipid subunit
	TC378198	Cinnamyl alcohol dehydrogenase
	CA731724	Glycosyltransferase
miR6220	CK211600, CK216047,CK214985, CK214076	High light protein
	TC377933	ATP dependent Clp protease ATP-binding subunit ClpX1
	TC433753	Proline-rich spliceosome-associated protein-like
	TC392962	Serine protease-like protein
	TC453352	Probable esterase PIR7A
miR818	TC390499, TC449066	S-adenosylmethionine decarboxylase

		proenzyme
	TC408578	Lipase class 3-like
	TC420068, CD936868, TC430395	Pathogenesis-related protein precursor
	TC445541	Wali2 protein
	TC452182	Glyoxalase II
	TC373115	Leucine zipper protein-like
	TC433753	Proline-rich spliceosome-associated protein-like
	TC377933	ATP dependent Clp protease ATP-binding subunit ClpX1

Target sequence: DFCI Index accession

APPENDIX S

Predicted Targets of Putative microRNAs of Bread Wheat 5D Chromosome Short Arm

microRNA	Target sequence	Target Protein
miR1117	BJ310907	30S ribosomal protein S5, chloroplast precursor
	CA723738	Aquaporin
	CK203951	Aspartate aminotransferase, cytoplasmic
	TC394273	Aspartokinase
	CK165300	AT4g15940/dl4011w
	TC408973, TC383766	Beta-carotene hydroxylase
	TC413428	Chloroplast methionine sulfoxide reductase B2 precursor
	CO347756	Cytochrome b5
	TC435744	Ferredoxin-sulfite reductase precursor
	BJ232689, TC391859	Initiator binding protein
	CJ675529	Nucleobase-ascorbate transporter 12
	TC405271	RNA recognition motif (RRM)-containing protein-like
	TC391048	UDP-glucOse dehydrogenase
miR1118	TC369361, CK196934, TC370181	Calmodulin TaCaM2-1
	TC379298	Geranylgeranyl hydrogenase
	TC418995	Glutathione S-transferase 2
	TC391749	Protein HVA22
	CV773860	Type 1 non specific lipid transfer protein precursor
miR1120	TC402657	Glyceraldehyde-3-phOsphate dehydrogenase, cytOsolic
	CJ668032	Leucine Rich Repeat family protein
	TC373115	Leucine zipper protein-like
	CJ572772	OJ000126_13.4 protein
	TC390499	S-adenOsylmethionine decarboxylase proenzyme
	TC449066	S-adenOsylmethionine decarboxylase proenzyme
miR1121	TC372427	Catalase-1
	TC405028	Extracellular invertase

	TC440824	O-methyltransferase family protein, expressed
	TC404730, TC389275	Peroxidase 6
	TC446821	Preprotein translocase secA subunit
	CJ839181	Ubiquitin carrier protein
miR1122	TC409078	40S ribOsomal protein S12
	CJ531707	Betanidin 6-O-glucOsyltransferase-like protein
	CD883901	Fusarium resistance protein I2C-5-like
	AL819672	Sodium/hydrogen exchanger
miR1125	CD454825	Plasma membrane proton ATPase
miR1127	TC434949	Sialyltransferase like protein
	BE516586	Calmodulin-like protein
miR1128	CK162453	Acyl-coenzyme A oxidase 2, peroxisomal precursor
	TC426247	AP-2 complex subunit sigma-1
	TC403387	At2g44820/T13E15.17
	TC454714	ATP sulfurylase
	CJ907078	Brain protein 44-like
	TC409696	Branched-chain-amino-acid aminotransferase-like protein 1
	BE516586	Calmodulin-like protein
	TC439689	Calreticulin-like protein
	TC369045	CBFIVd-B22
	DR731606	DEAD-box ATP-dependent RNA helicase 42
	BJ263423	Early light-inducible protein ELIP
	BQ838905	Early nodulin protein
	TC381299	Gamma-secretase subunit PEN2-like
	TC448466	Glycine-rich_protein_(Aa1-291) precursor
	CJ943226	Hydrolase, alpha/beta fold family protein, expressed
	TC415559	Malic enzyme
	CK168772	Monodehydroascorbate reductase
	CK198514	Peroxidase 6
	GH728396	Potential autophagy related protein-like
	BJ323295	Receptor protein kinase
	TC405876	Ring zinc finger protein-like
	TC434949	Sialyltransferase like protein
	TC388253	Speckle-type POZ protein-like
	CJ868604	Transcriptional activator-like

	GH726155	Type 1 non-specific lipid transfer protein precursor
	TC374504	UDP-D-glucOse epimerase 1
	TC369447	Zinc finger protein ZFP-like
miR1130	TC381627	Cinnamyl-alcohol dehydrogenase-like protein
	CD911932	Endonuclease-like
	TC395633	Ethylene receptor-like protein
	CA599831	Lipase (Class 3)-like protein
	CJ861664	PhOsphatidylinOsitol transfer-like
	TC453352	Probable esterase PIR7A
	TC378043	Seryl-tRNA synthetase family protein, expressed
	TC461034	Ubiquitin conjugating enzyme 7 interacting protein 4-like
miR1131	CD491034	3-phOsphOshikimate 1-carboxyvinyltransferase
	CA707384, CA678412	Chlorophyll a-b binding protein, chloroplast precursor
	TC431084	Metallothionein-like protein type 3
	TC372505	RibulOse bisphOsphate carboxylase/oxygenase activase A, chloroplast precursor
	CK211747, CK215758, CK213931	WCOR719
miR1133	TC454714	ATP sulfurylase
	CJ907078	Brain protein 44-like
	CK196934	Calmodulin TaCaM2-1
	BE516586	Calmodulin-like protein
	BQ838905	Early nodulin protein
	TC448466	Glycine-rich_protein_(Aa1-291) precursor
	CK198514	Peroxidase 6
	TC394164	PhOsphatidylinOsitol transfer protein, expressed
	TC405876	Ring zinc finger protein-like
	TC439651	Selenium-binding protein-like
	GH726155	Type 1 non-specific lipid transfer protein precursor
miR1135	TC433142	Alliin lyase-like
	TC390088	Coatomer subunit zeta-1
	CD931690	Cytochrome P450

	CD888735	Delta1-pyrroline-5-carboxylate synthetase
	TC374632	Fructan exohydrolase
	TC418338	GAD1
	TC456258	Importin beta-like protein
	TC374375, TC376089	Mitochondrial 2-oxoglutarate/malate translocator
	TC445105, TC394690, TC404007, TC441900, TC424561, TC401095	Monodehydroascorbate reductase
	TC383624	Peroxidase 6
miR1136	BJ263423	Early light-inducible protein ELIP
	TC405058	Protein kinase domain containing protein, expressed
miR1137	TC418521	Expansin-like A1 precursor
	CK197784	HGA1
miR1139	DR740890, DR740868, TC369138	At1g05520/T25N20_16
	TC405700	C3HC4 zinc finger containing protein
	TC386006	Cyclin-dependent kinases regulatory subunit 1
	TC391844	Gamma-TIP-like protein
	TC436537	General substrate transporter
	CJ636238	Glycine cleavage system H protein, mitochondrial precursor
	TC427924, TC414420	Kelch motif family protein, expressed
	TC424689, TC390083, TC391891, CA607350	L-lactate dehydrogenase A
	CK158705	Quinone reductase
	TC387148	SAR DNA binding protein
	TC429437	Senescence-associated protein 12
	TC397585	Transcription factor Myb1
	TC436930	WD-40 repeat protein-like
miR1436	TC377933	ATP dependent Clp protease ATP-binding subunit ClpX1
	TC369361, CK196934, TC370181	Calmodulin TaCaM2-1
	TC383543	Citrate synthase
	TC376766	DEAD-box ATP-dependent RNA helicase 42
	DR732284	ERN3
	TC440827	F-box domain containing protein-like
	TC418995	Glutathione S-transferase 2

	TC402657	Glyceraldehyde-3-phOsphate dehydrogenase, cytOsolic
	CV762246	GlycOsyltransferase
	TC452182	Glyoxalase II
	TC373115	Leucine zipper protein-like
	CJ563368	Lipid transfer protein-like
	DR732905	MIKC-type MADS-box transcription factor WM22A
	CJ572772	OJ000126_13.4 protein
	TC433753	Proline-rich spliceOsome-associated protein-like
	TC459951	Protein kinase domain containing protein, expressed
	BE425224	Receptor-like kinase 17 precursor
	DR732123	Regulatory protein of P-starvation acclimation response Psr1
	TC370176	Resistance-related receptor-like kinase
	TC437336	RibulOse bisphOsphate carboxylase large chain
	CJ557246	RNA pseudouridylate synthase family protein expressed
	TC390499, TC449066	S-adenOsylmethionine decarboxylase proenzyme
	TC453849	U2AF large subunit
	TC444038	Zinc finger CCCH domain-containing protein ZFN-like 1
miR1439	CJ936328	Alpha-L-arabinofuranOsidase/beta-D-xylOsidase isoenzyme ARA-I
	TC369361, TC370181	Calmodulin TaCaM2-1
	TC409271	CDK5 activator-binding protein-like
	TC383543	Citrate synthase
	TC394286	CTV.22
	TC376766, DR731536	DEAD-box ATP-dependent RNA helicase 42
	TC456061	Delta 1-pyrroline-5-carboxylate synthetase
	TC379298	Geranylgeranyl hydrogenase
	TC383916	Glutathione transferase
	TC452182	Glyoxalase II
	TC406001,TC413664	Histone H2B.1
	TC415559	Malic enzyme

	TC401095, TC424561, TC441900, TC404007, TC394690, TC445105	Monodehydroascorbate reductase
	TC459951	Protein kinase domain containing protein, expressed
	TC405876	Ring zinc finger protein-like
	CJ557246	RNA pseudouridylate synthase family protein expressed
	TC434687	Small GTP-binding protein
	TC459612	Wall-associated kinase 2
miR167	TC402134	ABP-1
	TC398445	Multidrug resistance associated protein 1
	CK193500	NADPH oxidoreductase homolog
	CK213113	Protochlorophyllide reductase A, chloroplast precursor
	TC395319	transcription factor jumonji (jmjC) domain-containing protein
miR1847	TC416401	Alpha tubulin-2A
	TC450471	F5I14.29/F5I14.29
	TC437514	F-box protein-like
	CK163755	Malic enzyme
	TC459671	Respiratory burst oxidase protein A
	TC401001	T1K7.26 protein
miR2118	TC451647	Alkaline invertase
	CK199587	Early nodulin 75-like protein
	TC439977, TC415030	GTP-binding protein-like
	TC422336	Kinase associated protein phOsphatase
	TC447958	Light stress-responsive one-helix protein-like
	CK212176	PhotOsystem 1 subunit 5
	TC371825	Serine/threonine protein phOsphatase
	TC403906	Triticain beta
miR3700	TC371315, TC371563,TC372626, TC437840	Polyubiquitin
	TC412324	P-type H+-ATPase
	TC409264	SET domain protein-like

miR395	TC401715, CV776184, TC455080, TC402080, TC441063, ,CK197311, CV761940	ATP sulfurylase
	TC370044	RibOsomal protein L19
	CA618183, CA734658, TC386520	SacIy domain containing protein, expressed
	CK193704	SucrOse transporter
miR482	TC425646, TC421071	NBS-LRR type RGA
	CA678894, TC393142, TC421789, TC410768	OJ000223_09.9 protein
	TC402759	RibOsomal L14 protein
	CO348589	Short-chain alcohol dehydrogenase
miR5021	TC392013	60S ribOsomal protein L30
	TC427967	BHelix-loop-helix transcription factor
	CD910148	BZIP transcription factor
	CD925597	Cupin family protein, expressed
	CK205372	Expansin EXPA12
	TC424354	FERONIA receptor-like kinase
	TC402816, TC387108, TC438021	GASA2-like protein
	TC428351	Glutathione transferase
	TC403644, TC407139	Gt-2
	TC426868	Lustrin A-like
	TC373827	Pistil-specific extensin-like protein precursor
	TC387438	SedoheptulOse-1,7-bisphOsphatase, chloroplast precursor (SedoheptulOse bisphOsphatase) (SBPase) (SED(1,7)P2ase)
	CA613725, TC385467	Serine/threonine-protein phOsphatase PP1
	TC421839	Sodium/hydrogen exchanger
	TC385179	WRKY transcription factor 28
miR5049	TC433193	1-aminocyclopropane-1-carboxylate oxidase
	TC394281	40S ribOsomal protein S15
	TC444618	Abscisic stress ripening-like protein
	TC414519	Acyl carrier protein 3, chloroplast precursor
	CJ531707	Betanidin 6-O-glucOsyltransferase-like protein
	TC416512	Citrate synthase

	TC394286	CTV.22
	TC436262	Cyclic nucleotide gated channel
	CJ727851	Cyclin-dependent kinase D-1
	CD931690	Cytochrome P450
	TC431549	Enhancer of rudimentary homolog
	TC435443	Expansin EXPB8
	TC418521	Expansin-like A1 precursor
	CD891662	F-box protein-like
	CA611067	Glutathione-S-transferase 28e45
	TC416752, TC438186	Harpin-induced protein 1 containing protein, expressed
	TC376269	High molecular mass early light-inducible protein HV58, chloroplast precursor
	TC413664	Histone H2B.1
	TC406957	Homeobox domain containing protein, expressed
	TC422449	InOsitol 1,3,4,5,6-pentakisphOsphate 2-kinase
	TC377619	Lecithin cholesterol acyltransferase
	CK215459	Low molecular mass early light-inducible protein HV90, chloroplast precursor
	TC438636	Mitochondrial phOsphate transporter
	TC376188	MYB20 protein
	TC380088	NBS-LRR type resistance protein-like
	CA711590	Nucellin-like aspartic protease
	TC398706	PG3
	TC449345	Phytepsin precursor (Aspartic proteinase) [Contains Phytepsin 32 kDa subunit
	TC382066, TC426391	Protein phOsphatase 2C
	TC370810	RadA-like protein
	CD903703	SGRP-1 protein
	TC423859	Short-chain dehydrogenase/reductase protein-like
	CJ530860	SNF7 protein-like
	AL819672	Sodium/hydrogen exchanger
	TC396713	SucrOse-phOsphate synthase 9
	TC423054	T6A9.6 protein
	TC432536	VMP3 protein
	TC438644	Wpk4 protein kinase
	TC452930	WRKY39
miR5067	TC374008	Calnexin
	CA676115	CI2C

	TC393687	Flavonoid 7-O-methyltransferase
	CK155004	Geraniol 10-hydroxylase
	TC422449	InOsitol 1,3,4,5,6-pentakisphOsphate 2-kinase
	TC371448	Methionyl-tRNA synthetase-like protein
	BJ257353	Peroxidase
	BJ307801	Transducin family protein-like
	TC438644	Wpk4 protein kinase
miR5068	CA605881	Acidic ribOsomal protein
	BE217043	Phenylalanine ammonia-lyase
miR5070	TC457231	Proline-rich protein
	TC440699	Translation initiation factor eIF5
miR5175	GH722323	10-deacetylbaccatin III-10-O-acetyl transferase-like
	TC444618	Abscisic stress ripening-like protein
	TC380076	BRI1-KD interacting protein 109
	TC439689	Calreticulin-like protein
	TC394286	CTV.22
	TC391404	Cysteine synthase (O-acetylserine sulfhydrylase) (O- acetylserine (Thiol)-lyase)
	DR731536	DEAD-box ATP-dependent RNA helicase 50
	TC391647	Oxidoreductase, 2OG-Fe oxygenase family protein, expressed
	TC398706	PG3
	CJ868604	Transcriptional activator-like
miR5180	AL816051	BARE-1 polyprotein
	TC425152	Peptidyl-prolyl cis-trans isomerase
	CJ608275	PhotOsystem II polypeptide
	CD903828	Starch branching enzyme I precursor
	TC413536	TIA-1 related protein
miR5181	TC387235	Citrate synthase
	TC374061, TC384192	Nuclear movement protein-like
	CJ596436	Translin family protein, expressed
	CJ963181	Zinc-finger motif
miR5203	TC383543	Citrate synthase
	CA647968	LEM3-like
	TC385465	Non-specific lipid-transfer protein
	CJ557246	RNA pseudouridylate synthase family protein expressed

	CJ553250	Serine carboxypeptidase family protein, expressed
	CJ530860	SNF7 protein-like
miR5205	TC444618	Abscisic stress ripening-like protein
	TC420918	Arf6/ArfB-family small GTPase
	TC377933	ATP dependent Clp protease ATP-binding subunit ClpX1
	AL821953	CHY zinc finger family protein, expressed
	CJ883403	Cysteine synthase
	TC385385	Exoglucanase precursor
	CD880846	Flowering promoting factor-like 1
	TC452182	Glyoxalase II
	CK211600, CK216047, CK214985, CK214076	High light protein
	TC389043	Malate dehydrogenase [NADP], chloroplast precursor
	DR732905	MIKC-type MADS-box transcription factor WM22A
	CN012529	Mitochondrial transcription termination factor-like protein
	TC398674	Phytoene dehydrogenase, chloroplast/chromoplast precursor
	CD879785	Probable protein ABIL1
	TC433753	Proline-rich spliceOsome-associated protein-like
	TC459951	Protein kinase domain containing protein, expressed
	TC406931	RibOsomal protein L7
	TC449066, TC390499	S-adenOsylmethionine decarboxylase proenzyme
	CD879878	Serine-threonine protein kinase
	TC395950	STF-1
	TC453849	U2AF large subunit
	TC383983	Utp14 protein, expressed
miR5387	TC437702	Histone H3.2
miR5568	TC397912	AdenOsine diphOsphate glucOse pyrophOsphatase precursor
	CJ936328	Alpha-L-arabinofuranOsidase/beta-D-xylOsidase isoenzyme ARA-I
	TC420918	Arf6/ArfB-family small GTPase
	CJ883403	Cysteine synthase
	TC401164	Fb14

	TC446402	Glutathione gamma-glutamylcysteinyltransferase 1
	TC452182	Glyoxalase II
	CK214076, CK214985, CK216047, CK211600	High light protein
	TC389043	Malate dehydrogenase [NADP], chloroplast precursor
	CN012529	Mitochondrial transcription termination factor-like protein
	TC398674	Phytoene dehydrogenase, chloroplast/chromoplast precursor
	CD879785	Probable protein ABIL1
	TC392962	Serine protease-like protein
	CD879878	Serine-threonine protein kinase
	TC395950	STF-1
	TC383983	Utp14 protein, expressed
miR6197	AL821953	CHY zinc finger family protein, expressed
	TC398842	Glutamine synthetase
	TC446402	Glutathione gamma-glutamylcysteinyltransferase 1
	TC380096	haloacid dehalogenase-like hydrolase family protein
	TC438131	Ice recrystallisation inhibition protein
	TC391197	Leucine Rich Repeat family protein, expressed
	TC369729	LRK14
	CA601255	Membrane bound O-acyl transferase MBOAT
	CK200742	Metallo-beta-lactamase-like
	CJ861664	PhOsphatidylinOsitol transfer-like
	TC442517	Protein HVA22
	TC440668	RibOsomal Pr 117
	CJ868604	Transcriptional activator-like
	CJ705099	Uncharacterized protein At1g55300.2
	TC383983	Utp14 protein, expressed
	TC419868	Zinc finger protein
miR6224	TC402657	Glyceraldehyde-3-phOsphate dehydrogenase, cytOsolic
miR834	TC400043	Chitinase
	CK213195	Histone H1
	DR733985	Auxin-repressed protein

miR845	TC399949	CRS2-associated factor 2, chloroplast precursor
	BQ902506	Triticain gamma
	TC436507	Hypersensitive response protein
miR950	TC400027	C2H2 zinc-finger protein
	TC441942	60S ribOsomal protein L19-like protein

Target sequence: DFCI Index accession

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